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Clarin-2 is essential for hearing by maintaining stereocilia integrity and function

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Abstract

Hearing relies on mechanically gated ion channels present in the actin-rich stereocilia bundles at the apical surface of cochlear hair cells. Our knowledge of the mechanisms underlying the formation and maintenance of the sound-receptive structure is limited. Utilizing a large-scale forward genetic screen in mice, genome mapping and gene complementation tests, we identified Clrn2 as a new deafness gene. The Clrn2Δclarinet/clarinet mice (p.Trp4* mutation) exhibit a progressive, early-onset hearing loss, with no overt retinal deficits. Utilizing data from the UK Biobank study, we could show that CLRN2 is involved in human non-syndromic progressive hearing loss. Our in-depth morphological, molecular and functional investigations establish that while it is not required for initial formation of cochlear sensory hair cell stereocilia bundles, clarin-2 is critical for maintaining normal bundle integrity and functioning. In the differentiating hair bundles, lack of clarin-2 leads to loss of mechano-electrical transduction, followed by selective progressive loss of the transducing stereocilia. Together, our findings demonstrate a key role for clarin-2 in mammalian hearing, providing insights into the interplay between mechano-electrical transduction and stereocilia maintenance.

Keywords: hair cells; mechanotransduction; mouse models; mutagenesis; stereocilia

Introduction

The process of hearing requires the transduction of sound wave-induced mechanical energy into neuronal signals. This process is achieved by the mechanosensitive inner ear hair cells located in the cochlea. These specialized sensory cells, named inner hair cells (IHCs) and outer hair cells (OHCs), have an array of actin-filled stereocilia protruding from their apical surface. Each hair cell stereocilia bundle is arranged as 3–4 rows in a highly ordered “staircase-like” structure, which is essential for function. Each taller stereocilium is connected to a shorter neighbour, in an adjacent row, by an extracellular tip link (Kazmierczak et al., 2007), with the upper end of the tip link extending from the side of a taller-row stereocilium to the tip of a shorter-row stereocilium, where it is tethered to the transduction channel complex. In response to sound-induced fluid movement within the inner ear, hair cell bundles are deflected towards the tallest stereocilia.
causing tension in the tip links, which opens the mechanically gated transduction channels, allowing the influx of K⁺ and Ca²⁺ ions into the hair cell, leading to depolarization and release of neurotransmitter (Corey & Hudspeth, 1983; Schwander et al., 2010). Components of the elusive transduction channel complex include LHFPIL tetraspan subfamily member 5 (LHFPIL5), transmembrane inner ear (TMIE) and transmembrane channel-like 1 (TMCL1) and TMCL2 (Kawashima et al., 2011; Kurima et al., 2015; Corns et al., 2016, 2017; Fettiplace, 2016; Beurg et al., 2018). All these proteins are reported to interact with protocadherin-15 (PCDH15), a component of the tip link, anchoring it to the stereocilia membrane (Xiong et al., 2012; Maeda et al., 2014; Zhao et al., 2014). The development and maintenance of the "staircase" stereocilia bundle, and the inter-stereociliary tip links, are therefore critical for auditory transduction and essential for hearing. Currently, our knowledge of the mechanisms underlying stereocilia bundle formation and maintenance is limited, and the precise molecular composition of the transduction channel complex remains elusive.

The Clarin (CLRN) proteins belong to a superfamily of small integral proteins with four alpha-helical transmembrane domains, which also includes Tetraspanins, Connexins, Claudins, Occludins and calcium channel gamma subunit-like proteins (Adato et al., 2002; Bonnet & El-Amraoui, 2012). Similarly, during a recent phenotype-driven ENU-mutagenesis screen undertaken at the MRC Harwell Institute, pedigree MPC169 was identified as containing mice with hearing impairment (Potter et al., 2016). In a G₃ cohort of 69 mice, 8 were found to have severely elevated auditory brainstem response (ABR) thresholds at 3 months of age (Fig 1A). A genome scan and subsequent single nucleotide polymorphism (SNP) mapping of affected (deaf) and unaffected (hearing) G₂ mice demonstrated linkage to a ~12-Mb region on Chromosome 5 (Fig EV1A). Whole-genome sequencing of an affected mouse identified a homozygous mutation within the critical interval consisting of a non-synonymous G-to-A transition at nucleotide 12 of the Clrn2 gene (ENSMUST0000053250). The Clrn2 mutation, confirmed using Sanger sequencing (Fig EV1B), leads to a tryptophan-to-stop (p.Trp4*) nonsense mutation in the encoded clarin-2 protein, a tetraspan-like glycoprotein with a class-II PDZ-binding motif (Fig 1B and C). We subsequently named this mutant clarinet and back-crossed the Clrn2clarinet allele to C57BL/6J for ten generations.

To confirm Clrn2clarinet is the causal mutation underlying the auditory dysfunction observed in clarinet mice, we first used a CRISPR/Cas9 approach to engineer a second Clrn2 mutant mouse model, named Clrn2del629. This allele consists of a 629 nucleotide deletion that encompasses exon 2 (ENSMUSE0000041986) of the Clrn2 gene, which encodes the second, and part of the third, transmembrane domains of clarin-2. As such, while the remaining exons 1 and 3 splice together and are in-frame, any translated protein is predicted to have reduced, or absent, function (Figs 1D and EV1C). Next, we undertook a complementation test crossing together these two Clrn2 mutant lines (Figs 1E and EV1D). ABR measurements, recorded in postnatal day 28 (P28) mice in response to click and tone-burst stimuli, showed that compound heterozygous (Clrn2clarinet/del629) mice display very elevated thresholds (> 80 decibel sound pressure level (dB SPL)) at all frequencies tested: 8, 16 and 32 kHz, whereas Clrn2clarinet/+ and Clrn2del629/+ mice exhibit thresholds comparable with those of wild-type (Clrn2+/+) littermates (< 40 dB SPL) (Figs 1E and EV1D), demonstrating the absence of a heterozygous auditory phenotype. Failure of complementation in Clrn2clarinet/del629 mice confirms the gene Clrn2 is essential for hearing.

Utilizing an unbiased forward genetic screen, we have identified an ENU-induced Clrn2 mutation as the cause of deafness in the clarinet mouse mutant (Clrn2clarinet). Moreover, we have employed Clrn2clarinet mice and a second CRISPR/Cas9-induced mutant (Clrn2del629) to investigate the requirement of clarin-2 in the auditory, vestibular and visual systems. While clarin-2 appears to have a nonessential role in the retina and vestibular apparatus, its absence leads to an early-onset progressive hearing loss. In addition, we identify that genetic variation at the human CLRN2 locus is highly associated with adult hearing difficulty in the UK Biobank Cohort. Expression of tagged clarin-2 in cochlear cultures shows enrichment of the protein in hair cell stereocilia. We demonstrate that clarin-2 is not required for the initial patterning, or formation, of the “staircase” stereocilia bundle, but instead is essential for the process of maintenance of the stereocilia bundle and mechano-electrical transduction. This study establishes a critical role for the tetraspan protein clarin-2 in the function of the mammalian auditory system.

Results

Clarin-2, a novel protein essential for mammalian hearing

During a recent phenotype-driven ENU-mutagenesis screen undertaken at the MRC Harwell Institute, pedigree MPC169 was identified as containing mice with hearing impairment (Potter et al., 2016). In a G₃ cohort of 69 mice, 8 were found to have severely elevated auditory brainstem response (ABR) thresholds at 3 months of age (Fig 1A). A genome scan and subsequent single nucleotide polymorphism (SNP) mapping of affected (deaf) and unaffected (hearing) G₂ mice demonstrated linkage to a ~12-Mb region on Chromosome 5 (Fig EV1A). Whole-genome sequencing of an affected mouse identified a homozygous mutation within the critical interval consisting of a non-synonymous G-to-A transition at nucleotide 12 of the Clrn2 gene (ENSMUST0000053250). The Clrn2 mutation, confirmed using Sanger sequencing (Fig EV1B), leads to a tryptophan-to-stop (p.Trp4*) nonsense mutation in the encoded clarin-2 protein, a tetraspan-like glycoprotein with a class-II PDZ-binding motif (Fig 1B and C). We subsequently named this mutant clarinet and back-crossed the Clrn2clarinet allele to C57BL/6J for ten generations.

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Utilizing the UK Biobank Cohort (Sudlow et al., 2015), a multi-phenotype study of 500,000 people aged between 40 and 69 years, we also sought whether genetic variation at the CLRN2 locus is related to self-reported human hearing difficulty. The association was performed using a case–control design (n = 250,389) based on answers to questions regarding participants’ self-assessed hearing ability and self-reported hearing difficulty in the presence of background noise. An association was tested between all 484 UK Biobank genotyped and imputed SNPs within 100 kb of the CLRN2 gene. Within this region, 36 SNPs were significantly associated with the hearing difficulty phenotype, including a cluster of five highly associated SNPs that lie within or very close to the CLRN2 gene (Fig 1F). Within the 20 most highly associated SNPs, the majority are either intronic or intergenic (Table EV1). The rs35414371 SNP with the highest association has a P-value of 1.60E-11 and lies just 2 kb downstream of the CLRN2 gene. The second most associated
SNP, rs13147559 (P = 1.70E-11), is in exon 2 of the CLRN2 gene at coding nucleotide position 337 (c.337, ENST00000511148.2). Presence of the ancestral allele (cytosine, c.337C) encodes for leucine (p.113Leu), whereas presence of the minor allele (guanine, c.337G) encodes for valine (p.113Val). As such, this SNP (c.337C > G) represents a missense variant (p.Leu113Val) within the predicted
transmembrane domain 2 of the clarin-2 protein (NP_001073296). In silico studies show that the leucine at position 113 is evolutionarily conserved across species. Furthermore, two prediction tools, PolyPhen-2 and MutationAssessor, suggest that substitution of a valine at this position might be detrimental to clarin-2 function returning scores of “possibly damaging” and “medium”, respectively.

Together, our findings indicate that clarin-2 is key to hearing in both mice and humans.

**Clarin-2 is essential for hearing function**

Clarin-2 displays 56% amino acid similarity with clarin-1, the Usher3A protein. Indeed, CLRN1 loss of function has been shown to cause progressive hearing loss, variable vestibular dysfunction and progressive retinitis pigmentosa, which prompted us to seek whether Clrn2 is a candidate Usher gene. RT-PCR analyses from wild-type P30 mice revealed the presence of Clrn2 transcripts in the inner ear (notably in the auditory hair cells) and the eye, but not in brain or muscle (Fig 2A and B). Thus, functional measurements were performed to characterize hearing, vestibular and visual phenotypes in clarinet mice.

To establish the onset and progression of auditory impairment in Clrn2+/−/clarinet mice, ABR measurements were undertaken at P16, which is just after the onset of hearing in mice (~P12), and longitudinally at P21, P28 and P42. At P16, Clrn2+/−/clarinet mice display elevated hearing thresholds (e.g. mean click threshold 64 dB SPL ± 15 SD) compared with their littermate controls (mean click threshold < 30 dB SPL ± 6 SD for Clrn2+/+ mice) (Fig 2C). At P21, Clrn2+/−/clarinet mice displayed increased auditory thresholds (mean click threshold 80 dB SPL ± 16 SD) compared with P16 Clrn2+/−/clarinet mice, and thresholds continue to increase by P28, and P42 (mean click threshold 89 dB SPL ± 5 SD; Fig 2D–F). To further assess cochlear function, distortion product otoacoustic emissions (DPOAEs) were measured in P28 Clrn2+/−/clarinet mice. Compared to their Clrn2+/+ and Clrn2+/+ littersmates, Clrn2+/−/clarinet mice have reduced DPOAEs (Fig 2G) suggesting impaired OHC function. These results show that lack of clarin-2 causes an early-onset hearing loss, characterized by a fast-progressive deterioration of hearing function likely affecting both inner and outer hair cells.

To assess for potential vestibular deficits, clarinet and control mice were subject to various tests, including platform, trunk-curl, contact righting and swim tests (Hardisty-Hughes et al., 2010). Regardless of the test employed, no overt difference between Clrn2+/−/clarinet mice (n = 12) and age-matched Clrn2+/+ control mice (n = 10) was observed at P28 or at P60, indicating normal balance function in young animals despite the absence of clarin-2 (Fig 3A; P > 0.05).

To investigate for possible vestibular deficits, clarinet mice, we measured the retinal-evoked potential responses, characterized by an initial negative deflection (the a-wave) followed by a positive peak (the b-wave), the amplitudes of which vary with light intensity. Functional electroretinogram (ERG) measurements, under scotopic (Fig 3B, D and E) or photopic (Fig 3C and F) conditions, indicated that rod and cone functions are normal in Clrn2+/−/clarinet mutant mice at both 3 months and 6–7 months. The ERG responses were almost normal in shape, with unaffected time-to-peak values for the a- and b-waves (Fig 3B and C). The amplitudes measured at the peak of both the a- and b-waves were also similar for control (Clrn2+/+ and Clrn2+/+ mice) at 6–7 months (a-wave, Fig 3D: 172 ± 14 and 207 ± 17, respectively; b-wave, Fig 3E: 299 ± 15 and 242 ± 39, respectively; photopic ERGs, Fig 3F: 61 ± 7 and 67 ± 8, respectively). Consistent with ERG findings, the overall laminar organization of the retina in control Clrn2+/+ and mutant Clrn2+/−/clarinet mice, examined on cryosections from mice aged 6–7 months, is normal, with normal retinal pigment epithelium, clearly distinguishable neuroretinal layers, and normal targeting and restriction of the short wavelength-sensitive opsins 1 (S opsin) and rhodopsin to the outer segment in blue cone and rod photoreceptor cells, respectively (Fig EV2A and B). No pyknotic nuclei, indicative of degenerating cells, were observed in any of the retinal cell layers, and TUNEL assays detected no apoptosis (Fig EV2C). The lba1-immunoreactive microglial cells in the retinas of Clrn2+/−/clarinet mice had features typical of the resting state similar to age-matched controls, including long thin neurites and lack of immunostaining in the photoreceptor cell-containing layer (Fig EV2D).

Together, our findings indicate that the absence of clarin-2 leads to an early-onset, progressive hearing loss, without overt retinal deficits.

**Clarin-2 is not required for the formation and proper polarization of the hair bundle**

To investigate the cause of hearing loss in Clrn2+/−/clarinet mice, and considering the previously reported disrupted organization of auditory hair bundles in neonatal Clrn1 mutant mice (Geller et al., 2009; Geng et al., 2009, 2012; Dulon et al., 2018), we used confocal and scanning electron microscopy to monitor the progression of hair cell stereocilia bundle development and maturation from birth (Figs 4 and 5).

At P6, towards the end of the first postnatal week, Clrn2+/−/clarinet cochlear OHC and IHC hair bundles have a cohesive appearance, with the expected V- and U-shape organization, respectively, compared to heterozygous controls (Fig 4A and B). Moreover, throughout Clrn2+/−/clarinet cocheae, stereocilin localizes as expected at the tips of the tallest stereocilia (Fig 4C and E, n = 3), indicating normal coupling between OHC stereocilia and the overlying tectorial membrane (Verpy et al., 2008). These data contrast with the grossly misshapen auditory OHC hair bundles exhibited by Clrn1 mutant mice (Geller et al., 2009; Geng et al., 2009; Dulon et al., 2018) (see also Fig 4D). By P8, scanning electron microscopy shows Clrn2+/−/clarinet mutants do not exhibit any gross patterning defects, or differences in the overall number of OHC and IHC bundles compared to controls (Fig 4F and G). Mutant hair bundles throughout the cochlea still displayed the 3–4 rows of stereocilia arranged in a regular “staircase-like” pattern as in age-matched control mice (see Fig 5A and B). Also, cochlear OHC apical circumferences have lost their immature rounded shape, to acquire a non-convex form moulded to the V-shape of the overlying hair bundle (arrowheads in Fig 4A, B, F and G).

The first notable morphological defect in Clrn2+/−/clarinet mice was observed in the auditory hair cells of the cochlea towards the end of the first postnatal week. Detailed analyses at P8 revealed that the shortest row stereocilia in mutant OHCs appear shorter than those of Clrn2+/+ littersmates (Fig 5A and C). In addition, the tips of
the middle and shortest row stereocilia in Clrn2<sup>-/-</sup> mutant IHCs are less prolate compared to Clrn2<sup>+/+</sup> littermates, instead displaying a rounded appearance (Fig 5B). Focusing on the second stereocilia row in both IHCs and OHCs, we used cochlear mid-turn electron micrographs from control and clarin-2-deficient hair bundles at P8 to score prolateness. At least 80 tip images per genotype—3 animals per genotype, 3 bundles per animal—we found a high prevalence of a rounded shape of the stereocilia from clarinet mice, as compared to age-matched wild-type mice, where the normal prolate shape is far more common ($P < 0.005$ for all cases, $\chi^2$) (Fig EV3). To further characterize the bundle architecture, we measured the heights of OHC and IHC stereocilia (tallest, middle and shortest rows) within individual hair cell bundles from the mid-region of the cochlea ($\geq 9$ stereocilia per bundle, $\geq 2$ bundles per animal, 3 animals per genotype). At P8 in OHC bundles, the average heights of stereocilia in all three rows are significantly shorter in Clrn2<sup>-/-</sup> mice relative to Clrn2<sup>+/+</sup> littermates (tallest $-13\%$, middle $-25\%$, shortest $-52\%$) (Fig 5C). However, at P8 in
IHC bundles we do not identify any significant differences in the average heights of stereocilia between Clrn2\(^{clarinet/clarinet}\) mutants and Clrn2\(^{+/+}\) controls in any row (Fig 5D). By P16 in OHC bundles, the average height of stereocilia in all three rows continue to be shorter in Clrn2\(^{clarinet/clarinet}\) mice relative to Clrn2\(^{+/+}\) littermates (tallest 9%, middle 25%, shortest 87%), with many short row stereocilia now missing (Fig 5C), and by P28, this row is entirely absent (Fig 5E, left panels). At P16 in IHC bundles, similar to P8 we do not identify any differences in the average heights of IHC stereocilia between Clrn2\(^{clarinet/clarinet}\) mutants and Clrn2\(^{+/+}\) controls in any row (Fig 5D). However, the tips of the middle and shortest row IHC stereocilia in Clrn2\(^{clarinet/clarinet}\) mice continue to display a rounded appearance, unlike the pronounced prolate shape observed in Clrn2\(^{+/+}\) littermates (Fig 5B). Furthermore, the height of the shorter-row stereocilia is more variable with some very short stereocilia measured (Fig 5D). Moreover, by P28 the heights of the middle and short row are visibly more variable in Clrn2\(^{clarinet/clarinet}\) mice, and missing short row IHC stereocilia are evident (Fig 5E, right panels).

These data suggest that while clarin-2 is dispensable for pattern- and establishment of the “staircase” bundle in young postnatal hair cells, it is critical for the maintenance of the transducing stereocilia in functionally mature inner and outer hair cells.

Clarin-2 is targeted to the hair bundles of the hair cells

To analyse the expression profile of Clrn2, whole-cochlea RNA extracts were prepared from wild-type mice at different embryonic and postnatal timepoints and utilized for quantitative RT–PCR (qRT–PCR) analysis of Clrn2 transcripts. This shows that the relative abundance of Clrn2 transcripts in the cochlea is stable from late embryonic stages to P12 (the onset of hearing in mice), but

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**Figure 3.** Clarin-2 is dispensable for balance and vision.

A Vestibular behavioural tests (swim tests, contact-righting, trunk-curl and platform). The Clrn2\(^{clarinet/clarinet}\) mice (red, P28, n = 5, and P60, n = 7) have no vestibular dysfunction, displaying similar performances to age-matched control Clrn2\(^{+/+}\) mice (grey, P28, n = 3, and P60, n = 7) (Student’s t-test was used for the platform assay, and the Pearson’s Chi-squared test for other experiments). Being similar, the values at P28 and P60 were combined.

B–F Electroretinogram (ERG) measurements from control Clrn2\(^{+/+}\) (grey) and mutant Clrn2\(^{clarinet/clarinet}\) (red) mice. Each trace in (B, C) is representative of an ERG response from the eye of age-matched Clrn2\(^{+/+}\) and Clrn2\(^{clarinet/clarinet}\) mice, showing no significant difference in a- or b-wave amplitudes. (D–F) The lack of change in ERG amplitude responses in Clrn2\(^{clarinet/clarinet}\) mice (aged 3 or 6–7 months), regardless of the test conditions: scotopic (D, E) or photopic (F) indicates normal photoreceptor kinetics and no change in the sensitivity of photoreceptor cells. The data shown are mean ± SEM. (NS) indicates a statistically nonsignificant difference (P > 0.1, Student’s t-test).
thereafter increases (Fig EV4A). Consistent with this finding, in silico analyses of the expression of Clrn2 at different inner ear developmental and adult stages using the gEAR portal (umgear.org) reveal that while the Clrn2 transcript is lowly expressed in the newborn inner ear and early postnatal stages, it is readily detected in P15 single-cell and adult-sorted hair cells (Liu et al., 2014, 2018; Ranum et al., 2019). In addition, the expression of Clrn2 is detected in zebrafish hair cells (Steiner et al., 2014; Erickson & Nicolson, 2015). Interestingly, Clrn2 transcripts were detected also in the auditory cortex (A1) and increased in levels between P7 and adult mice (Guo et al., 2016). The Clrn2 expression in both IHCs and OHCs was confirmed by RT–PCR on isolated auditory hair cells from P15 mice (Fig 2B). Regarding protein localization, several attempts were made to immunodetect endogenous mouse clarin-2, including raising an antibody against mouse clarin-2. While this purified antibody was able to recognize overexpressed clarin-2 in transfected cells, it does not detect endogenous clarin-2 despite various tests using cochlear whole mounts from different postnatal stages, and under various conditions of fixation and antigen-retrieval (Fig EV4B–E). Therefore, an

Figure 4. Normal architecture of cochlear hair bundles in clarinet mice.

A, B Confocal microscopy images of whole-mount preparations of mid-basal cochlear sensory epithelia from Clrn2clarinet+/+ (A) and Clrn2clarinet/clarinet (B) P6 mice immunostained for actin. Despite lack of clarin-2, the developing sensory epithelium of mutants is similar to that of heterozygous controls.

C–E Confocal microscopy images of whole-mount preparations of cochlear sensory epithelia from Clrn2clarinet+/+ (C), Clrn1–/– (D) and Clrn2clarinet/clarinet (E) P6 mice immunostained for stereocilin (green) and actin (red). Unlike the fragmented immunostaining in Clrn1–/– mice (mirroring the fragmentation of the hair bundle), stereocilin immunostaining in Clrn2clarinet+/+ mice reflects the normally V-shaped bundles of OHCs, similar to Clrn2clarinet+/+. OHCs.

F, G Representative scanning electron micrographs of the sensory epithelium of Clrn2clarinet+/+ (F) and Clrn2clarinet/clarinet (G) P8 mice, showing no apparent differences in the gross patterning of IHCs and OHCs.

Data information: Arrowheads in (A, B) (white), (F, G) (black) illustrate the convex shape of the apical circumference of OHCs. Scale bars: 2 μm (A–E), 10 μm (F, G).
injectoporation approach (Xiong et al., 2014) was utilized to deliver a GFP-tagged clarin-2 expression construct to P2 cochlear cultures (see Fig 6A). After incubation, the injectoporated organs of Corti were fixed and co-stained with an anti-GFP antibody to detect the clarin-2 fusion protein, and phalloidin to visualize the stereocilia. In contrast to supporting cells, where the GFP-tagged clarin-2 is distributed diffusely throughout the cytoplasm (Fig 6B and C), in auditory hair cells, clarin-2 is enriched in the apical stereocilia (Fig 6D–F).

**Lack of clarin-2 causes selective defects in the mechano-electrical transduction machinery**

To study molecular underpinnings of the bundle stereocilia remodelling in *clarinet* mice, we explored the distribution of myosin VIIa (used as a hair cell marker, Fig EV5A) and some selected proteins key to stereocilia growth and hair bundle organization (Figs 7 and EV5B–D). Considering the clarin-2 C-terminal class-II PDZ-binding motif, we investigated whether the absence of clarin-2 might...
interfere with the subcellular distribution of PDZ-containing proteins, namely whirlin, PDZD7 and harmonin. These deafness
defective PDZ-containing adaptor proteins have been shown to anchor stereocilia integral membrane proteins to the underlying
cytoskeleton (Boeda et al., 2002; Mburu et al., 2003; Grati et al., 2012). Within stereocilia, the subcellular distribution of whirlin and
PDZD7 immunostainings were identical in 2019 and B, and EV5B; proper shaping of the auditory hair bundles, respectively (Figs 7A
and B, and EV5B). Conversely, IHC stereocilia measurements at P8 and P16 did not show a difference between Clrn2+/+ and Clrn2+/fli
in: for the shortest row at P8, Clrn2+/+, 0.6188 ± 0.0944 pm (n = 27); Clrn2+/fli/clarnet in: e.g. for the shortest row at P8, Clrn2+/+.
0.2948 ± 0.1029 pm (n = 27) (P = 0.0001, Mann–Whitney ranks comparison), and at P16, Clrn2+/+, 0.579 ± 0.0617 pm (n = 27);
Clrn2+/fli/clarnet in: 0.0762 ± 0.0162 pm (n = 27) (P = 0.0001, Mann–Whitney ranks comparison). Note the amount of value points equal to zero at P16 (0)
Distribution of individual stereocilia measures across genotypes at P8 and P16. (C) The OHC stereocilia measurements at both P8 and P16 (C) show a significant
difference in height of Clrn2+/+ stereocilia compared to Clrn2+/fli/clarnet in: e.g. for the shortest row at P8, Clrn2+/+, 0.6188 ± 0.0944 pm (n = 27);
Clrn2+/fli/clarnet in: 0.2948 ± 0.1029 pm (n = 27) (P = 0.0001, Mann–Whitney ranks comparison). Measurements were performed using IHC,
indicating normal coordination of synaptic body components and functional IHC ribbon synapses.

Lack of clarin-2 disrupts hair cell transducer currents in auditory hair cells

Harmonin-b relocation to the upper link link density has been measured in: e.g. for the shortest row at P8, Clrn2+/+, 0.8808 ± 0.1753 pm (n = 27);
Clrn2+/fli/clarnet in: 0.9313 ± 0.1217 pm (n = 27) (P = 0.2144, unpaired t-test); and at P16, Clrn2+/+, 0.6784 ± 0.1171 pm (n = 23);
Clrn2+/fli/clarnet in: 0.6063 ± 0.1855 pm (n = 26) (P = 0.2436, Mann–Whitney ranks comparison). **P < 0.01, ***P < 0.0001, NS P > 0.05.

E Pseudo-coloured scanning electron micrographs of individual OHC (left panels) and IHC (right panels) hair bundles from
Clrn2+/fli/clarnet mutants have only two rows of OHC stereocilia, and the middle row of stereocilia is less uniform in height compared to controls. The middle and
short rows of stereocilia in Clrn2+/fli/clarnet IHC bundles appear fewer in number, and heterogeneous in height.

Data information: Scale bars, 2 μm.

Figure 5. Sensory hair cell bundle patternning and measurements in clarinet mice.

A, B Pseudo-coloured scanning electron micrographs of individual outer and inner hair cell bundles from clarinet mice at P8 and P16, showing that gross morphology of
OH and IHC bundles is similar between Clrn2+/+ and Clrn2+/fli/clarnet mice. Representative images from the mid-region of the cochlear spiral are shown, and
close-up views illustrate the three full rows, tallest (red), middle (blue) and short (yellow), of stereocilia in IHC and OHC hair bundles. At P8 and P16 (upper panels
in A and B), the three stereocilia rows are observed in Clrn2+/fli/clarnet; OHCs (A) and IHCs (B), although the shortest and middle rows of IHC stereocilia appear less
prolate compared to controls.

C, D Distribution of individual stereocilia measures across genotypes at P8 and P16. (C) The OHC stereocilia measurements at both P8 and P16 (C) show a significant
difference in height of Clrn2+/+ stereocilia compared to Clrn2+/fli/clarnet in: e.g. for the shortest row at P8, Clrn2+/+, 0.6188 ± 0.0944 pm (n = 27);
Clrn2+/fli/clarnet in: 0.2948 ± 0.1029 pm (n = 27) (P = 0.0001, Mann–Whitney ranks comparison), and at P16, Clrn2+/+, 0.579 ± 0.0617 pm (n = 27);
Clrn2+/fli/clarnet in: 0.0762 ± 0.0162 pm (n = 27) (P = 0.0001, Mann–Whitney ranks comparison). Note the amount of value points equal to zero at P16 (0)

We also investigated the distribution of the harmonin-b isoform, a core component in mechano-electrical transducer (MET) transduction
machinery (Grillet et al., 2009; Michalski et al., 2009), which has been shown to directly bind to actin filaments anchoring the apical-most tip link component, cadherin-23, to the stereocilia
underlying cytoskeleton (Kazmierczak et al., 2007). In wild-type mice, between P1 and P5, harmonin-b localization switches from
stereocilia tips at this stage in: e.g. for the shortest row at P8, Clrn2+/+, 0.8808 ± 0.1753 pm (n = 27); Clrn2+/fli/clarnet in: 0.9313 ± 0.1217 pm (n = 27) (P = 0.2144, unpaired t-test); and at P16, Clrn2+/+, 0.6784 ± 0.1171 pm (n = 23);
Clrn2+/fli/clarnet in: 0.6063 ± 0.1855 pm (n = 26) (P = 0.2436, Mann–Whitney ranks comparison). **P < 0.01, ***P < 0.0001, NS P > 0.05.

E Pseudo-coloured scanning electron micrographs of individual OHC (left panels) and IHC (right panels) hair bundles from clarinet mice at P8.
Clrn2+/fli/clarnet mutants have only two rows of OHC stereocilia, and the middle row of stereocilia is less uniform in height compared to controls. The middle and
short rows of stereocilia in Clrn2+/fli/clarnet IHC bundles appear fewer in number, and heterogeneous in height.

Data information: Scale bars, 2 μm.
of MET channels was similar between Clrn2clarinet/+ and Clrn2clarinet/clarinet OHCs (Fig 8D and E). Because the MET current reverses near 0 mV, it became outward when excitatory bundle stimulation was applied during voltage steps positive to its reversal potential (Fig 8A–C). At positive membrane potentials (+99 mV), the larger resting MET current (Fig 8A and B, arrowheads), which is due to an increased open probability of the transducer channel resulting from a reduced driving force for Ca\(^{2+}\) influx (Crawford et al., 1989; Corns et al., 2014), was also similar between the two genotypes (Fig 8D). Similar findings were also observed in basal-coil OHCs (Fig 8E) and apical-coil IHCs (Fig 8F–H), with a significant reduction in the maximum MET current in Clrn2clarinet/clarinet mutants (P < 0.0001 for both hair cell types), but similar resting open probability, between Clrn2clarinet/+ and Clrn2clarinet/clarinet mice.

Overall, these data show that clarin-2 is required for normal MET current in developing cochlear hair cells.

Clarin-2 is required for the functional differentiation of IHCs, but not OHCs

In the mouse cochlea, the onset of adult-like characteristics in OHCs occurs at around P8 with the expression of the negatively activated K\(^+\) current I\(_{\text{K,n}}\) (Marcotti & Kros, 1999) carried by the KCNQ4 channel (Kubitsch et al., 1999). Potassium currents in apical-coil OHCs from P22 clarinet mice were elicited by applying a series of depolarizing voltage steps in 10 mV increments from /C0 144 mV (holding potential was /C0 84 mV). We found that adult OHCs from Clrn2clarinet/+ and Clrn2clarinet/clarinet mice express the same complement of K\(^+\) currents (Fig 9A–C). The size of I\(_{\text{K,n}}\) was also similar

Figure 6. Expression of clarin-2 in the hair bundle of auditory hair cells.

A Schematic representation of the auditory sensory organ, illustrating the positioning of the electrode used for injectoporation of GFP-tagged clarin-2 construct into cochlear supporting and hair cells.

B–F Top and side views of representative images of supporting cells (asterisk) and hair cells expressing clarin-2. In supporting cells, GFP-tagged clarin-2 (green) was distributed diffusely throughout the cytoplasm (B, C). By contrast, in all injectoporated hair cells, the majority of clarin-2 was observed in the apical stereocilia (D–F). Arrows indicate position of the hair bundle stereocilia. Scale bars, 2 µm.
between the two genotypes. These results indicate that absence of clarin-2 does not influence the acquisition of the adult-like basolateral membrane properties of OHCs.

Different to the OHCs, the onset of adult-like characteristics in IHCs occurs at around P12 (Kros et al., 1998; Marcotti, 2012). The IHC functional maturation is achieved by the down-regulation of immature-type currents (e.g. Na\(^+\) current and the small conductance Ca\(^{2+}\)-activated K\(^+\) current carried by SK2 channels) and inward rectifier (K1) currents) and expression of \(I_{K,n}\) and the fast-activating large-conductance Ca\(^{2+}\)-activated K\(^+\) current (\(I_{K,f}\): carried by BK...
channels) (Marcotti et al., 2003). Initially, we measured the size of the total outward $K^+$ current at 0 mV in P22 IHCs and found that it was similar between Clrn2+/clarinet- controls (11.6 ± 1.6 nA, $n = 4$) and Clrn2+/clarinet- mutants (10.7 ± 1.0 nA, $n = 5$). For these experiments, currents were elicited by applying depolarizing voltage steps in 10 mV incremental from -124 mV up to +30 mV, starting from the holding potential of -84 mV. However, a close inspection of the time-course of current activation showed some differences, which were evaluated in more detail by delivering a voltage protocol that allowed the evaluation of the both $k_o$ and $k_o$ (Fig 9D-F). IHCs were held at -64 mV and subjected to depolarizing voltages in 10 mV incremental from -144 mV to +30 mV, starting from the holding potential of -84 mV. We found that while Clrn2+/clarinet+ IHCs exhibit both $k_o$ and $k_o$, Clrn2+/clarinet- IHCs fail to show the above currents and instead express $k_i$ (Fig 9D-F), which is characteristic of a pre-hearing IHC (Marcotti et al., 1999). The physiological consequence of the above abnormalities was that adult Clrn2+/clarinet+ IHCs did not acquire the fast-graded voltage responses to stimulation normally present in mature cells (Fig 9G and H) and, in some cases, retained the ability to fire an initial Ca$^{2+}$-dependent action potentials (Fig 9I) that is characteristic of immature cells (Kros et al., 1998; Marcotti et al., 2003). We found that immature-type currents were not down-regulated in Clrn2+/clarinet+ IHCs, and as such, their functional differentiation into mature cells was prevented.

**Discussion**

We demonstrate that Clrn2 is a novel deafness gene required for maintenance of transducing stereocilia in the sensory cochlear hair cells. Our results suggest that the absence of clarin-2 leads to an early-onset hearing loss in clarinet mice, which is moderate-to-severe at P16, but rapidly progresses to profound hearing loss after P21. Moreover, our morpho-functional and molecular studies of clarin-2-deficient mice demonstrate this protein is critically required for late-stage cochlear hair bundle maintenance and function. In particular, we show that while clarin-2 is dispensable for the acquisition of polarized and assembled stereocilia bundles, the protein is essential for maintaining bundle integrity and normal sound-induced mechanoelectrical transduction.

Hair bundle formation and polarization is a multi-step process with three main phases reported in the mouse—initial (E15-P0), intermediate (P1-P5) and final (P6-P15) stages (Lefevre et al., 2008). During phase 1, growth of the stereocilia rows is uniform, whereas in phase 2 differential elongation leads to the staircase pattern, with concomitant regression of supernumerary stereocilia in the mature hair bundle (Kaltenbach et al., 1994). Previous work has shown that clarin-1 is critically involved in formation of properly shaped IHC and OHC hair bundles (Geller et al., 2009; Geng et al., 2009, 2012; Dulon et al., 2018). Importantly, no such disorganization occurs in the absence of clarin-2. We show that during cochlear development and maturation, the OHC and IHC hair cell bundles in clarin-2-deficient mice appear to grow normally up to ~P5, developing their characteristic “staircase” architecture, and typical V- and U-shape organization, respectively. Indeed, by P6 almost all cochlear OHC apical circumferences have lost their immature rounded shape, to acquire a non-convex form moulded to the V-shape of the overlying hair bundle (arrowheads in Fig 4A, B, F and G). Together, these findings suggest that polarity cues (Kelly & Chen, 2007; Ezan & Montcouquiol, 2013), as well as the interactions and cellular remodelling between supporting and sensory hair cells necessary for normal patterning of the auditory organ (Keller et al., 2000; Etournay et al., 2010), occur normally in the absence of clarin-2. Furthermore, the persistence of immunostaining for PDZD7, a key member of the ankle link complex at the stereocilia base, correlates with the proper shaping of the V- and U-shaped hair bundles (Grati et al., 2012). Also, the persistence of whirlin (Fig 7B) and EPS8 (Fig EV5D), two proteins key for actin polymerization, at the tips of the differentiating stereocilia (Mburu et al., 2003; Manor et al., 2011; Zampini et al., 2011) are consistent with normal stereocilia elongation up to at least P8 in the absence of clarin-2. Moreover, stereocilin is properly targeted to the distal tips of the tallest row stereocilia, indicating normal anchoring of these OHC stereocilia into the overlying tectorial membrane. Thus, we conclude that the first molecular and structural steps of hair bundle morphogenesis (initial and intermediate phases) are not affected in the absence of clarin-2.

Our qRT–PCR analyses show a postnatal increase of Clrn2 transcripts in wild-type mouse cochleae, indicating a potential key function after the onset of hearing (~P12 in mice). We show that upon injection into cochlear organs, clarin-2 localizes to stereocilia. Interestingly, only in hair cells does GFP-tagged clarin-2 target to apical surface cell membranes, indicating that additional hair cell-specific co-factors are likely required for proper
subcellular targeting of clarin-2 to the plasma membrane of stereocilia. In the Clrm2clarinet/Clarinet mice, despite normal shape organization, the absence of clarin-2 leads to a progressive reduction in height of the middle and shortest row stereocilia, which is evident first in OHCs by P8, and then later in IHCs at P16. It is noteworthy that an abnormal shortening of mechanotransducing stereocilia has also been reported in mice deficient for several components of the mechanoelectrical transduction machinery, namely the TMC1/TMC2 channel complex (Kawashima et al., 2011), TMIE (Zhao et al., 2014), LHFPILS (Xiong et al., 2012), and sans or cadherin-23 (Caberlotto et al., 2011) and PCDH15 (Pepermans et al., 2014). Furthermore, Velez-Ortega and colleagues recently showed that reducing mechanoelectrical transduction currents in wild-type mouse or rat hair cells, using pharmacological channel blockers or disruption of tip links, leads to reduction in the height of the middle and shortest row “transducing” stereocilia (Velez-Ortega et al., 2017). Thus, it is possible that the stereocilia phenotype observed in clarinet mutant mice is a downstream consequence of a defect in mechanoelectrical transduction. Indeed, the onset of regression of the mechanotransducing stereocilia, occurring in the absence of clarin-2, is concomitant with the loss of normal MET responses, which are already identifiable by P6-7 at both the molecular and functional levels. First, from P8 onwards, instead of the normal prolate-shaped tips of the transducing stereocilia evident in wild-type mice, which is believed to be a result of the traction force exerted by the tip link on the stereocilium apical membrane (Rzadzinska et al., 2004; Prost et al., 2007), clarin-2-deficient transducing
stereocilia display round, oblate apical stereocilia wedges (Fig EV3). Second, the number of PDZD7 immunoreactive spots, essentially between the short and middle transducing stereocilia, is decreased in the absence of clarin-2, indicating loss of cytoskeleton–membrane crosslinkers that probably accompanies the regression of the short stereocilia row (morphologically visible at later stages, about P16 in OHCs). Third, the actin-binding USH1C protein harmonin-b (Boeda et al., 2002), a core component of the MET machinery (Grillet et al., 2009; Michalski et al., 2009), is mislocalized in the absence of clarin-2 at P6. A similar
Clrn1 to the upper attachment point of the tip link, facing the tips of the adjacent shorter stereocilia actin core. Finally, detailed electrophysiological recordings in IHCs and OHCs at P6-8 showed that in the absence of clarin-2 the properties of the MET channel are normal, as shown by the similar resting open probability at positive membrane potentials, indicative of reduced adaptation in the unstimulated bundle from both genotypes (Corns et al., 2014). However, the maximal current is significantly reduced by approximately 50% in mutant IHCs and OHCs, indicating there are less available functional channels. This reduction could result from missing tip links and/or lack of sustaining tension on still present tip links, which would also be consistent with our finding of non-prolate middle and shortest row stereocilia. The presence of a functional MET current in pre-hearing IHCs, but not OHCs, has been shown to be crucial for their functional maturation into fully functional sensory receptors (Corns et al., 2018).

In humans, recessive CLRN1 mutations cause Usher syndrome type IIIa (USH3A, MIM276902), characterized by post-lingual, progressive hearing loss, and variable balance and vision loss deficits (Adato et al., 2002). Interestingly, almost all USH3A patients develop normal speech, and a number display only mild-to-moderate hearing threshold elevation at the time of hearing loss diagnosis, at an age of 30–40 years (Ness et al., 2003). This contrasts with the phenotype of Clrn1 knockout mice, where lack of clarin-1 has been shown to cause an early profound hearing loss (Geller et al., 2009; Geng et al., 2009, 2012; Dulon et al., 2018). Additionally, Clrn1<sup>−/−</sup> mice exhibit profound hearing loss by P25, even though USH3A patients with the CLRN1<sup>−/−</sup> mutation display post-lingual progressive hearing loss (Ness et al., 2003; Geng et al., 2012). Furthermore, Clrn1 knockout mice do not exhibit overt retinal deficits, and so do not model this aspect of USH3A. Our characterization of the clarinet mutant establishes that lack of clarin-2 results in a progressive, early-onset hearing loss in mice, with no overt retinal deficits. However, given the interspecies difference in phenotypic presentation observed with Clrn1, we cannot exclude the possibility that pathogenic CLRN2 mutations in humans might give rise to an Usher syndrome-like phenotype. Moreover, our studies show that clarin-2, unlike clarin-1 which is required during embryonic stages, is dispensable for the patterning and establishment of the “staircase” bundle in young postnatal hair cells. This finding supports our hypothesis that clarin-2 has an important role in functionally mature cochlear hair cells to maintain proper integrity of the transducing stereocilia. Accordingly, we investigated the UK Biobank cohort and found that genetic variation at the human CLRN2 locus is highly associated with adult hearing difficulty in this cohort.

Our analysis revealed a cluster of SNPs that lie within, or close to, the CLRN2 gene, which are significantly associated with an adult hearing difficulty phenotype. Within the 20 most highly associated SNPs, the majority are either intronic or intergenic (Table EV1). As such, it is probable that these do not directly affect CLRN2 expression. Instead, these SNPs are likely in linkage disequilibrium with an, as yet unidentified, causal variant(s) within the UK Biobank population cohort. However, the second most associated SNP (rs13147559) is located within the CLRN2 gene coding sequence (c.337C>G), with presence of the minor allele causing a leucine-to-valine missense variation at codon 113 (p.Leu113Val). This leucine residue, based on comparison to the 3D modelling prediction of hsCLRN1 (Gyorgy et al., 2019), is located within the second transmembrane domain of hsCLRN2 and is evolutionarily conserved across species. While this variation involves two hydrophobic amino acids that possess similar structures, valine does have a shorter side chain. Furthermore, prediction tools suggest that this substitution might be detrimental to protein function returning scores of “possibly damaging” and “medium”. Interestingly, similar substitutions located in the highly conserved transmembrane domains of presenilin, encoded by the gene PSEN1, have been reported in patients with Alzheimer’s disease. These missense variants (p.Leu250Val and p.Leu153Val) have been proposed to interfere with the helix alignment of the transmembrane domain altering protein optimal activity, thus accounting for disease expression (Furuya et al., 2003; Larner, 2013). However, additional studies are needed to determine whether the hsCLRN2 p.Leu113Val missense variant is causal of, or merely associated with, the adult hearing difficulty trait. Perhaps it may be that “mild” CLRN2 hypomorphic mutations, such as p.Leu113Val may represent, is likely to predispose to a progressive, late-onset hearing loss phenotype. Conversely, it is possible that more pathogenic CLRN2 mutations may elicit a more severe, early-onset hearing loss phenotype. There are examples of this, for instance TMPRSS3, encoding transmembrane protease serine 3, has been reported to cause severe-to-profound prelingual hearing loss (DFNB10) as well as progressive hearing impairment with post-lingual onset (DFNB8) due to differential pathogenic mutations (Gao et al., 2017). Of note, a recent work by Gopal S. and colleagues reported a recessively inherited non-syndromic sensorineural hearing loss in a consanguineous Iranian family caused by a CLRN2 mutation that results in a missense mutation in the encoded protein (Gopal et al., 2019). Affected patients develop post-lingual moderate-to-profound hearing loss with no indication of balance or vision deficits. Altogether, while mutations in CLRN1 unambiguously lead to USH3A, current findings suggest that CLRN2 mutation most likely causes non-syndromic hearing loss. However, additional cases need to be identified to clarify the genotype-phenotype relationship between the impaired extent of activity of the mutated clarin-2 protein, the age of onset, the severity and the extent of the disease phenotype. Nonetheless, our study demonstrates the utility of interrogating human large cohort study data as a means to help validate candidate genes arising from forward genetic, or whole-genome sequencing, screens.

**Conclusion**

Clrn2<sup>clarinet</sup> mice are ENU-induced mutants that exhibit early-onset sensorineural hearing loss, associated with a nonsense mutation in the encoded tetraspan protein clarin-2. Utilizing these mice, we demonstrate that clarin-2 is required for the maintenance of stereocilia bundle morphology, and show that its loss leads to decreased mechano-electrical transduction and progressive hearing impairment. Moreover, utilizing data from the UK Biobank study, CLRN2 is identified as a novel candidate gene for human non-syndromic progressive age-related hearing loss. Our study of the clarinet mouse
Materials and Methods

Mice

The clarinet mutant was identified from the MRC Harwell Institute phenotype-driven N-ethyl-N-nitrosourea (ENU) Ageing Screen (Potter et al, 2016). In this screen, ENU-mutagenized C57BL/6J males were mated with wild-type “sighted C3H” (C3H.Pde6b+) females (Hoeltzer et al, 2008). The resulting G1 males were crossed with C3H.Pde6b− females to produce G2 females, all of which were screened for the Cdh23<sup>abl</sup> allele (Johnson et al, 2006). Cdh23<sup>+/−</sup> G2 females were then backcrossed to their G1 fathers to generate recessive G3 pedigrees, which entered a longitudinal phenotyping pipeline. Auditory phenotyping comprised clickbox testing at 3, 6, 9 and 12 months of age and ABR at 9 months of age. Initial linkage was determined using SNP mapping (Tepnel Life Sciences), delineating a critical interval containing the clarinet mutation on Chromosome 5. Whole-genome sequencing was undertaken using DNA from an affected G3 mouse (Oxford Genomics Centre, Wellcome Trust Centre for Human Genetics, University of Oxford) and putative lesions validated by standard PCR and Sanger sequencing. <i>Clrn2<sup>clarinet</sup></i> carrier mice were subsequently backcrossed to C57BL/6J for ten generations until congenic. The <i>Clrn2<sup>del629</sup></i> mutant line was generated on a C57BL/6N background by the Molecular and Cellular Biology group at the Mary Lyon Centre (MLC), MRC Harwell Institute using CRISPR-Cas9 genome editing (Mianne et al, 2016; Codner et al, 2018) (Table EV2). Within the MLC, all mice were housed and maintained under specific pathogen-free conditions in individually ventilated cages, with environmental conditions as outlined in the Home Office Code of Practice. Animals were housed with littermates until weaned and then housed with mice of the same gender and of similar ages, which was often their littermates. Both male and female animals were used for all experiments.

Animal procedures at the MRC Harwell Institute and University of Sheffield were licensed by the Home Office under the Animals (Scientific Procedures) Act 1986, UK, and additionally approved by the relevant Institutional Ethical Review Committees. Animal procedures at the Institut Pasteur were accredited by the French Ministry of Agriculture to allow experiments on live mice (accreditation 75-10-01, issued on 6 September 2013 in appliance of the French and European regulations on care and protection of the Laboratory Animals (EC Directive 2010/63, French Law 2013-118, 6 February 2013)). Protocols were approved by the veterinary staff of the Institut Pasteur animal facility and were performed in compliance with the NIH Animal Welfare Insurance #A5476-01 issued on July 31, 2012.

Association analysis of human hearing with <i>CLRN2</i> variation in the UK Biobank Cohort

The cohort used for the human association analysis consisted of 500,000 UK Biobank (UKBB) participant (Sudlow et al, 2015) with “White British” ancestry. Samples with excess heterozygosity, excess relatedness and sex discrepancies were identified and removed prior to analysis. Samples were genotyped on one of two arrays; 50,000 samples were genotyped on the Affymetrix UK BiLEVE Axiom array, while the remaining 450,000 were genotyped on the Affymetrix UK Biobank Axiom<sup>®</sup> array. The two arrays shared 95% coverage resulting in > 800,000 genotyped SNPs. Imputation was carried out centrally by UKBB, primarily using the HRC reference panel and IMPUTE2 (Howie et al, 2009, 2011). Analysis in this study was conducted with version 3 of the UK Biobank imputed data.

For association testing, cases and controls were defined based on participants’ responses to questions regarding hearing ability. Briefly, participants who answered YES to both “Do you have any difficulty with your hearing?” and “Do you find it difficult to follow a conversation if there is background noise (such as TV, radio, children playing)?” were defined as cases, n = 102,832. Those who answered NO to the same questions were defined as controls. Any individuals who said they used a hearing aid were removed from the control group. Finally, individuals below the age of 50 years of age were removed from the controls to ensure a similar age distribution to cases, resulting in a sample with n = 163,333 for the control group.

A linear mixed models approach was used to test for association for all SNPs within 100 kb of the <i>CLRN2</i> gene using BOLT-LMM v.2 (Loh et al, 2018) for the association analysis, which corrects for population stratification and within-sample relatedness. In addition, analysis was adjusted for age, sex, UK Biobank genotyping platform and UK Biobank PCs1-10. For quality control, SNPs were filtered based on the two thresholds: (i) minor allele frequency > 0.01 (ii) INFO score > 0.7. Individuals with < 98% genotype call rate were removed. Following genomic quality control filters and selection for White British samples, association analysis was performed on the remaining 87,056 cases and 163,333 controls. To adjust for multiple testing, the Bonferroni-adjusted significance threshold for this analysis is set at 0.0009 based on calculating the effective number of independent SNPs within this region (n = 55) (Li et al, 2012).

Utilization of the UK Biobank Resource was conducted under Application Number 11516.

Auditory phenotyping

Auditory brainstem response (ABR) tests were performed using a click stimulus and frequency-specific tone-burst stimuli (at 8, 16 and 32 kHz) to screen mice for auditory phenotypes and investigate auditory function (Hardisty-Hughes et al, 2010). Mice were anesthetized by intraperitoneal injection of ketamine (100 mg/ml at 10% v/v) and xylazine (20 mg/ml at 5% v/v) administered at the rate of 0.1 ml/10 g body mass. Once fully anesthetized, mice were placed on a heated mat inside a sound-attenuated chamber (ETS Lindgren) and recording electrodes (Grass Telefactor F-E2-12) were placed subdermally over the vertex (active), right mastoid (reference) and left mastoid (ground). ABR responses were collected, amplified and averaged using TDT System 3 hardware and BioSig software (Tucker Davies Technology, Alachua, FL, USA). The click stimulus consisted of a 0.1-ms broadband click presented at a rate of 21.1/s. Tone-burst stimuli were of 7-ms duration including rise/fall gating using a 1-ms Cos2 filter, presented at a rate of 42.5/s. All stimuli were presented free-field to the right ear of the mouse,
starting at 90 dB SPL and decreasing in 5 dB increments until a threshold was determined visually by the absence of replicable response peaks. For graphical representation, mice not showing an ABR response at the maximum level tested (90 dB SPL) were recorded as having a threshold of 95 dB SPL. These mice/thresholds were included when calculating genotype average thresholds. All ABRs were performed blind to genotype, to ensure thresholds were obtained in an unbiased manner. Mice were recovered using 0.1 ml of anaesthetic reversal agent atipamezole (Antisedan™, 5 mg/ml at 1% v/v), unless aged P16, when the procedure was performed terminally.

Distortion product otoacoustic emission tests were performed using frequency-specific tone-burst stimuli from 8 to 32 kHz with the TDT RZ6 System 3 hardware and BioSig RZ software (Tucker Davis Technology, Alachua, FL, USA) software. An ER10B+ low-noise probe microphone (Etymotic Research) was used to measure the DPOAE inside the tympanic membrane. Tone stimuli were presented via separate MF1 (Tucker Davis Technology) speakers, with f1 and f2 at a ratio of f2/f1 = 1.2 (L1 = 65 dB SPL, L2 = 55 dB SPL). Mice were anaesthetised via intraperitoneal injection of ketamine (100 mg/ml at 10% v/v), xylazine (20 mg/ml at 5% v/v) and acepromazine (2 mg/ml at 8% v/v) administered at a rate of 0.1 ml/10 g body mass. Once surgical anaesthesia was confirmed by the absence of a pedal reflex, a section of the pinna was removed to enable unobstructed access to the external auditory meatus. Mice were then placed on a heated mat inside a sound-attenuated chamber (ETS Lindgren), and a pipette tip containing the DPOAE probe assembly was inserted into the ear canal. In-ear calibration was performed before each test. The f1 and f2 tones were presented continuously, and a fast-Fourier transform was performed on the averaged response of 356 epochs (each ~21 ms). The level of the 2f1-2f2 DPOAE response was recorded and the noise floor calculated by averaging the four frequency bins either side of the 2f1-2f2 frequency.

Clrn2 expression in tissues and isolated auditory hair cells

To allow analysis of the Clrn2Deldel29 allele, whole cochlear ducts were collected from P21 (+/− 1 day) Clrn2+/-, Clrn2+/del629 and Clrn2del629/del629 littermate mice, and stored in RNA later Stabilising Solution (Inviironet) at −20°C until processed. Total RNA was extracted using TRIzol Reagent (Invitrogen) and used as template for retrotranscription (Superscript One-Step RT-PCR system (Invitrogen). For tissue-specific Clrn2 expression studies, the primers employed were as follows: Clrn2-F1 (ATGCCCTGATGGTTCAAAAAG)/Clrn2-R1 (TCACAAAGGTGACCGAAGATCGC), and a β-actin control: β-actin-F (ACCTGACACTACCTCAT)/β-actin-R (AGACAGACTGTTGTTCGGC). For hair cell-type specific Clrn2 expression, the primers employed were as follows: Clrn2-F2 (GGGACGCCAGATCCAATTTT)/Clrn2-R2 (ACTCCACCTGGAGGACCAT), with hair cell-specific controls: Otoferlin (IHC positive control) Otof-F (CATCGAGTGTGCAGGAAAGG)/Otof-R (ACCTGACACACGACATCAGA); and Oncomodulin (OHC positive control) Ocm-F (CGGCCCTGCAGAATGCCAA)/Ocm-R (GCTTCAGGGGGACTTTGAT). PCR products were separated by electrophoresis on 2% agarose gels.

Behavioural tests

Multiple behavioural tests were used to assess the vestibular function of clarinet mice, as described previously (Hardisty-Hughes et al., 2010). In the platform test, mice were placed on a small platform (7 × 7 cm, at a height of 29 cm) and the time on the platform was recorded over a period of 2 min. The contact righting test consisted of placing a mouse in a closed transparent tube and determining whether it was able to successfully regain standing position upon a 180° rotation of the tube (score 1) or fail (score 0). For the swim test, each mouse was placed in a container filled with water at 22–23°C and given a score determined as follows: score 0 = normal swimming; score 1 = irregular swimming; score 2 = immobile floating; score 3 = underwater tumbling.

Electroretinogram response measurements

To measure electroretinograms (ERGs), animals were kept in the dark to adapt to darkness overnight as previously described (Michel et al., 2017). Each mouse was anesthetized with a mixture of ketamine (80 mg/kg, Axience, France) and xylazine (8 mg/kg, Axience, France), and placed over a warming pad to maintain body temperature at 37°C. Their pupils were dilated with tropicamide (Mydriaticum; Théa, Clermont-Ferrand, France) and phenylephrine (Neosynephrine; Europhtha, Monaco). The cornea was locally anesthetized with oxybuprocaine hydrochloride (Théa, Clermont-Ferrand, France). Upper and lower lids were retracted to keep eyes open and bulging. Retinal responses were recorded with a gold-loop electrode brought into contact with the cornea through a layer of lubrithal (Decrah, France), with needle electrodes placed in the cheeks and back used as reference and ground electrodes, respectively (Yang et al., 2009). The light stimuli were provided by an LED in a Ganzfeld stimulator (SIEM Bio-médicale, France). Responses were amplified and filtered (1 Hz-low and 300 Hz-high cut-off filters) with a one-channel DC/AC amplifier. One level of stimulus intensity (8 cd.s/m2) was used for scotopic ERG recording. Each of the response obtained was averaged over five flash stimulations. Photopic cone ERGs were recorded in a rod-suppressing background light of 20 cd.s/m2, after a 5-min adaptation period. An 8 cd.s/m2 level of stimulus intensity was used for the light-adapted ERGs. Each cone photopic ERG response presented was averaged over ten consecutive flashes.
Immunolabelling

For the synaptic labelling experiments, mice were culled by cervical dislocation and inner ears were fixed in 4% paraformaldehyde (PFA) in PBS for 1 h at room temperature (RT). Post-fixation, ears were fine dissected to expose the sensory epithelium then permeabilized using 0.1% Triton X-100 in PBS for 10 min at RT. Samples were blocked in 5% donkey serum (Sigma) for 1 h at RT and immunolabelled with primary antibodies overnight at 37°C. To enable detection, samples were incubated with fluorescein-coupled secondary antibodies for 1 h at 37°C then stained with DAPI (1:2,500, Thermo Fisher) for 5 min at RT. Samples were mounted onto slides in SlowFade® Gold (Life Technologies) and visualized using a Zeiss LSM 710 fluorescence confocal microscope. Primary antibodies: rabbit anti-Ribeye (Synaptic Systems; 192103; 1:200); mouse anti-GluR2 (Millipore; MABN1189; 1:200). Secondary antibodies: Alexa Fluor® donkey anti-rabbit 568 (Invitrogen; 1:200); Alexa Fluor® donkey anti-mouse 488 (Invitrogen; 1:500).

For all other immunofluorescence experiments, samples were processed as previously described (Michel et al., 2017). Briefly, for cochlear whole-mount preparations, micro-dissected, fixed mouse organs of Corti (4% PFA in PBS, pH 7.4 for 1 h at RT) were rinsed, then blocked by incubation in PBS supplemented with 20% normal goat serum and 0.3% Triton X-100 for 1 h at RT. After incubation with primary antibodies in PBS 1% bovine serum albumin overnight at 4°C, samples were rinsed in PBS then incubated with specific secondary antibodies (and phalloidin for actin staining when required) for 1 h at RT. They were then immersed in DAPI (Sigma) for nuclear labelling and rinsed before mounting using Fluorosave (Calbiochem, La Jolla, CA).

To test the occurrence of apoptosis in the retina, cryosections from control and clarinet mice were analysed using the in-situ Cell Death Detection Kit, Fluorosecin (Roche), according to the manufacturer’s instructions.

To detect clarin-2 protein, the cDNA encoding amino acid residues 159–232 (VKFHDLTERIANFQERLQOFVVFVEEQYEEFSFVIC VASASAHAAANLVVQIPSQLEIKTMKMEAVTYPDILY) of mouse Cltn2 were amplified by PCR, cloned into pRSET (Life Technologies) and transformed into BL21(DE3)pLysS competent cells (Life Technologies) for protein production. The fusion protein was purified using cobalt chloride-charged chelating sepharose fast flow resin (GE Healthcare Life Sciences) and used to immunize rabbits (Covalab). The antiserum were purified by affinity chromatography using the fusion protein (antigen) coupled to SulfoLink resin (Pierce), according to the manufacturer’s instructions. We checked the specificity of the affinity-purified antibodies by immunofluorescence using transfected cells and mouse organs of Corti (clarinet samples were used as negative controls). The purified homomade, but not commercial, anti-clarin-2 antibodies did detect both GFP- and FLAG-tagged clarin-2 in transfected cells (Fig EV4B-D). However, repeated attempts to detect endogenous clarin-2 in the mouse auditory sensory organ at different postnatal stages, under various conditions of fixation and antigen-retrieval, were unsuccessful. Similar observations were made using commercially available rabbit polyclonal anti-clarin-2 antibodies: the anti-clarin-2 from ProteinTech (1:100, 239941-AP) and from Atlas (HPA042407; 1:100). The absence of specific immune-detection in hair cells could reflect a low level of endogenous expression (Fig EV4).

To detect PDZD7, we used a newly generated homemade polyclonal rabbit antibody. It is derived against a mouse PDZD7 fusion protein (amino acid 2–83, accession number NP_001182194.1) and has been validated in transfected cells and mouse organs of Corti. The following other primary antibodies were used: rabbit anti-myosin VIIa (1:500), rabbit anti-harmonin-b (1:50) and rabbit anti-whirlin (1:100) (Sahly et al., 2012). Other primary antibodies were used: rabbit anti-stereoclin (1:150) (Verpy et al., 2008), mouse anti-EPS8 (1:200; 610144; BD Biosciences) mouse and rabbit anti-CtBP2 (1:200; Goat polyclonal, Santa Cruz, USA; SC-5966) to detect the ribbon protein ribeye, rabbit anti-GluR2/3 (1:200; Millipore), rabbit anti-opsin, blue (1:100; AB5407, Merck-Millipore), mouse anti-Iba1 (1:200; MABN92, Merck-Millipore), mouse anti-rhodopsin (1:500; MAB5316, Merck-Millipore), mouse anti-FLAG2 (1:120, F3165, Sigma-Aldrich) and rabbit anti-GFP (1:250, Invitrogen). The following specific secondary antibodies were used: ATTO 488-conjugated goat anti-rabbit IgG (1:500, Sigma-Aldrich) and ATTO 500 goat anti-mouse IgG antibody (1:500, Sigma-Aldrich). ATTO 565 phalloidin (1:700; Sigma-Aldrich) was also used to label F-actin.

Samples were imaged at RT with a confocal microscope (LSM 700; Carl Zeiss) fitted with a Plan-Apochromat 63× NA 1.4 oil immersion objective from Carl Zeiss. To quantify the positioning of the harmonin-b immunoreactive area relative to the tip of the taller stereocilium, we used Imagel software (NIH). Quantification was made using IHC, rather than OHC, bundles as it is easier to define individual stereocilia, allowing accurate measurements of the distance of the harmonin-b immunoreactive spot relative to the tip of the stereocilium. For each clarinet (n = 4) and wild-type (n = 4) mouse, confocal images of 8–10 IHCs with well-preserved tallest stereocilia were considered for measurements. For each cell, the distance value taken into consideration is an average value from 3 stereocilia of the same cell: for each stereocilium, we measure the distance between the stereocilium tip to the centre of harmonic-b-immunoreactive spot located on the side of the same stereocilium. Measurements were analysed using Student’s t-test.

Scanning electron microscopy

Mice were euthanized by cervical dislocation, and inner ears were removed and fixed in 2.5% glutaraldehyde (TAAB Laboratories Equipment Ltd.) in 0.1 M phosphate buffer (Sigma-Aldrich) overnight at 4°C. Following decalcification in 4.3% EDTA, cochleae were sub-dissected to expose the sensory epithelium then “OTO processed” with alternating incubations in 1% osmium tetroxide (TAAB Laboratories Equipment Ltd.) in 0.1 M sodium cacodylate (Sigma-Aldrich) and 1% thio-carbohydrazide (Sigma-Aldrich) in ddH2O. Ears were dehydrated through a graded ethanol (Fisher Scientific) series (25–100%) at 4°C and stored in 100% acetone (VWR Chemicals) until critical point drying with liquid CO2 using an Emetech K850 (EM Technologies Ltd.). Ears were mounted onto stubs using silver paint (Agar Scientific), sputter coated with palladium using a Quorum Q150R S sputter coater (Quorum Technologies) and visualized with a JSM-6010LV Scanning Electron Microscope (JEOL).

To analyse prolactenes of the stereocilia tips between control and clarin-2-deficient hair bundles, scanning electron micrographs were utilized and small square selections framing the tips of individual
second row stereocilia were delimited and extracted to create an array of tip images using ImageJ (Fiji). Consistency between the images captured was maintained by ensuring the side of the square selection was parallel to the distal edge of the stereocilium, and ensuring that a constant distance was kept from the upper edge. Stereocilia from OHC bundles were imaged at a ~45° angle from the perspective plane. All images were taken to scale after calibration to the scanning electron microscope software-generated scale bar. The image array for each genotype was then loaded as a pseudo z-stack and a median-intensity z-projection generated. For each projection, at least 80 tip images per genotype were used—3 animals per genotype, 3 bundles per animal, with all bundles from the mid-region of the cochlea. These projections were processed using auto-tone and then pseudo-coloured in Adobe Photoshop, before a perimeter outline was drawn using Adobe Illustrator in order to show the shape of the median Z-projected stereocilia tip (see Fig EV3).

To assess stereocilia heights, at least three ears (one ear per mouse) were analysed for each genotype at each time point. Duplicate images of the middle turn (180° to 360°) of the cochlea were taken, with a 5° tilt between them. IHC bundles were imaged at 8,000× magnification and OHC bundles at 15,000× magnification, all at a constant working distance of 20 μM. Three different bundles were analysed per animal, with up to nine different measurements taken per bundle: three measurements from the tallest row of stereocilia, three from the middle row and three from the shortest row (if present). IHC and OHC measurements were obtained using ImageJ software (NIH) and corrected using a pseudo-eucentric tilting approach (Bariani et al., 2005). A single measure \(x_1\) (e.g. length of the tallest row of one stereocilium) was taken on a first micrograph and measured again \(x_2\) on the corresponding 5°-tilted repeat micrograph. Perpendicular countermeasures \(y_1\) and \(y_2\) to \(x_1\) and \(x_2\) were also taken in every instance. Later, countermeasures were fed onto equation (1), in order to estimate uncertainty \(\xi\) due to plane rotation. Each pair of tilted-coupled measures \(x_1\) and \(x_2\) was then fed onto equation (2), along with the uncertainty estimate \(\xi\) from equation (1), thus obtaining a close approximation \(\hat{\xi}\) of the true measure of the structures investigated.

\[
\begin{align*}
\xi &= \frac{(\Delta y_1 \cos \Delta \phi + (2y_1(y_1 - \Delta y_1)/d) \sin \Delta \phi)}{(1 + y_1(y_1 - \Delta y_1)/d^2 \sin \Delta \phi) + (\Delta y/d) \cos(2\Delta \phi)}; \\
\hat{\xi} &= \frac{2d - 2\Delta z_2 \cos \Delta \phi}{d/x_1 + d/x_2},
\end{align*}
\]

where, \(\xi\) = uncertainty estimate; \(y_1, y_2\) = perpendicular countermeasures to measures \(x_1, x_2\); \(\Delta y\) = arithmetic difference of countermeasures \(y_1\) and \(y_2\); \(\Delta \phi\) = tilting angle (5°); \(d\) = working distance (20 μM); \(x_1, x_2\) = tilted paired-measures of structure of interest; \(\hat{\xi}\) = estimate of true size of structure of interest.

**Clrn2 expression in whole cochlea**

Total RNA samples were extracted from wild-type C57BL/6J mouse whole cochleae at E17.5 (embryonic day 17.5), P4, P8, P12, P16 and P28 using a Direct-zol RNA MiniPrep Kit (Zymo Research). Five separate samples were prepared for each age, comprising whole-cochlea mouse RNA extracted from both ears. In each case, quintuplicate samples from independent mice were analysed for each stage. Complementary DNAs were generated from 1 μg total RNA using the High Capacity cDNA synthesis kit (Applied Biosystems) following the manufacturer’s instructions. Amplifications were performed using TaqMan Gene Expression Assays with Fast Universal PCR Master Mix. A β-actin primer/probe set (MM00469690_g1) was used as an internal control, and a custom TaqMan assay was designed to allow the specific amplification of the *Clrn2* transcripts using the following forward and reverse primers, and TaqMan Gene Expression Assay: *Clrn2*-F 5′-AAGATGTC CACTTGCCCAAC-3′, *Clrn2*-R 5′-GACCAGGTTCCTTGCTTC-3′, Mm03990594_m1. PCRs were run in triplicate in the Applied Biosystems real-time PCR device (7500 Fast Real-Time PCR System) in 20-μl reactions containing 10 μl Fast TaqMan Master Mix, 1 μl TaqMan assay, 4 μl dH2O and 5 μl complementary DNA (5 ng/μl), using the following cycles: 95°C for 20 s and 40 cycles at 95°C for 3 s and 60°C for 30 s. Amplification data were recorded and relative quantification performed using the 7500 software version 2.0.6 software (Applied Biosystems) comparing threshold cycles (Ct). *Clrn2* mRNA levels were first normalized to *Actin* (ACT = Ct*Actin* – Ct*Clrn2* at each age, and changes in expression relative to P4 were calculated as 2−(ΔCt/ΔCtP4).

**Injectoporation**

The injectoporation experiments of cochlear explants were performed as previously described (Xiong et al., 2014), with a few modifications. In brief, cochlear were dissected from P2 wild-type mice, cut into 4–6 pieces and cultured for 6 h in DMEM/F12 medium with 100 ng/μl ampicillin. Next, adherent cochlear explants were placed between two platinum wire electrodes (Surepure Chemetals) and a patch pipette (2 μm diameter) placed between the second and third row of OHCs was used to deliver the plasmid (1 μg/μl in 1× HBSS) to the hearing organ. The pipette and electrode were positioned using an Axioscope 2 Carl Zeiss microscope with a 40× objective (Olympus) and two micromanipulators (Sutter MPC-200). To trigger plasmid entry into cells, 3 square-pulses with a magnitude of 60 V (15 ms length, 1-s intervals) were applied, using an ECM 830 electroporator (Harvard Apparatus). Organs of Corti were cultured for another 12 h in DMEM/F12 and fixed in 4% PFA for 1 h before immunostaining. Samples were analysed at RT with a confocal microscope (LSM 700; Carl Zeiss) fitted with a Plan-Apochromat 63× NA 1.4 oil immersion objective from Carl Zeiss.

**Electrophysiology**

Electrophysiological recordings were made from apical-coil OHCs and IHCs of *clarinet* mice aged P6-22. A number of basal-coil OHCs were also investigated at P7-8. Cochleae were dissected in normal extracellular solution (in mM): 135 NaCl, 5.8 KCl, 1.3 CaCl2, 0.9 MgCl2, 0.7 NaH2PO4, 5.6 glucose, 10 HEPES-NaOH. Sodium pyruvate (2 mM), MEM amino acids solution (50X, without l-Glutamine) and MEM vitamins solution (100X) were added from concentrates (Fisher Scientific, UK). The pH was adjusted to 7.5 (308 mosmol/kg). The dissected cochlea were transferred to a microscope samples.
chamber, immobilized with a nylon mesh (Corns et al., 2016) and continuously perfused with a peristaltic pump using the above extracellular solution. The organs of Corti were viewed using an upright microscope (Leica DMLMF, Germany; Nikon FN1, Japan) with Nomarski optics (x60 or x63 objectives).

MET currents were elicited by stimulating the hair bundles of OHCs and IHCs using a fluid jet from a pipette (tip diameter 8–10 μm) driven by a piezoelectric disc (Corns et al., 2014). The pipette tip of the fluid jet was positioned near to the bundles to elicit a maximal MET current. Mechanical stimuli were applied as 50 Hz sinusoids (filtered at 0.25 kHz, 8-pole Bessel) with driving voltages of ±40 V. MET currents were recorded with a patch pipette solution containing (in mM): 106 Cs-glutamate, 20 CsCl, 3 MgCl2, 1 EGTA-CsOH, 5 Na2ATP, 0.3 Na2GTP, 5 HEPES-CsOH, 10 sodium phosphocreatine (pH 7.3). Membrane potentials were corrected for the liquid junction potential (LJP) of −11 mV, measured between electrode and bath solutions.

Patch clamp recordings were performed using an Optopatch (Cairn Research Ltd, UK) amplifier. Patch pipettes were made from soda glass capillaries with a typical resistance in the extracellular solution of 2–3 MΩ. In order to reduce the electrode capacitance, patch electrodes were coated with surf wax (Mr Zoggs SexWax, USA). Basolateral membrane recordings were performed with an intracellular solution containing (in mM): 131 KCl, 3 MgCl2, 1 EGTA-KOH, 5 Na2ATP, 5 HEPES-KOH, 10 Na2-phosphocreatine (pH 7.3; osmolality ~296 mmol/kg). Data acquisition was controlled by pClamp software using Digidata 1440A boards (Molecular Devices, USA). Recordings were low-pass filtered at 2.5 kHz (8-pole Bessel), sampled at 5 kHz and stored on computer for off-line analysis (Origin: OriginLab, USA). Membrane potentials in voltage clamp were corrected for the uncompensated residual series resistance and for a LJP of ~4 mV measured between electrode and bath solutions. All recordings were performed at RT.

**Statistical analysis**

Unless stated, data were analysed using Student’s two-tailed t-test (two study groups) or one-way ANOVA followed by Tukey’s post-hoc test (three study groups) using Graphpad Prism. Graphs are presented as mean ± SD, unless stated, and P < 0.05 indicates statistical significance. Using data obtained from the original MPC169 cohort, we used GraphPad StatMate to carry out power calculations to determine sample size in an unpaired t-test using the standard deviation of the measured hearing thresholds, a significance level of P = 0.01 (two-tailed) and a power of 95%. As such, an effect size was estimated using real data corresponding to the clarinet hearing loss phenotype comparing hearing thresholds of wild-type and homozygous littermates. This determined that a cohort size of ≥4 mice/genotype would be sufficient to detect an auditory threshold difference. All phenotyping was performed blind to genotype, and no data were excluded from analysis.

**Availability of materials**

Clrn2<sup>−/−</sup> and Clrn2<sup>+/−</sup> mice are available on request from the MRC Harwell Institute.

**The paper explained**

**Problem**

Hearing loss is a very prevalent condition that can result from environmental causes, genetic predisposition or an interaction of both. Over the last two decades, while much progress has been made in understanding the genetic bases of congenital and early-onset hearing loss, we have only just begun to elaborate upon the genetic landscape of age-related hearing loss. Going forward, increased knowledge of the genes and molecular pathways required for the maintenance of hearing function will likely provide opportunities for the design of therapeutic approaches to prevent progressive hearing loss.

**Results**

Utilizing a forward genetic screen in mice, we identified a mutation within the Clrn2 gene as the cause of hearing loss in the clarinet mutant. Loss of the encoded tetraspan-like protein clrn-2 leads to an early-onset, progressive hearing loss. Interestingly, analysis of a large cohort of patients with adult hearing difficulties from the UK Biobank study we identify Clrn2 as a novel candidate gene for human non-syndromic progressive age-related hearing loss. Our morphological, molecular and functional investigations of the clarin-2-deficient mice establish that while the protein is not required for the initial formation of cochlear sensory hair cell stereocilia bundles, it is critical for maintaining normal bundle integrity and functioning. In the differentiating hair bundles, lack of clarin-2 leads to loss of mechanoelectrical transduction, followed by selective progressive loss of the transducing stereocilia.

**Impact**

Integrated mouse and human approaches continue to elaborate upon our understanding of the genetic mechanisms required for mammalian hearing. In this study, we establish that Clrn2 is a novel deafness gene associated with progressive hearing loss in both mice and humans, and as such, severe loss-of-function Clrn2 mutations should be considered in the aetiology of human autosomal recessive hearing loss. Moreover, our findings demonstrate a key role for clarin-2 in mammalian hearing, providing insights into the interplay between mechanoelectrical transduction and stereocilia architecture maintenance.

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