Defective Gpsm2/G\(\alpha_{i3}\) signalling disrupts stereocilia development and growth cone actin dynamics in Chudley-McCullough syndrome

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Mutations in GPSM2 cause Chudley-McCullough syndrome (CMCS), an autosomal recessive neurological disorder characterized by early-onset sensorineural deafness and brain anomalies. Here, we show that mutation of the mouse orthologue of GPSM2 affects actin-rich stereocilia elongation in auditory and vestibular hair cells, causing deafness and balance defects. The G-protein subunit G\(\alpha_{i3}\), a well-documented partner of Gpsm2, participates in the elongation process, and its absence also causes hearing deficits. We show that Gpsm2 defines an \(~200\) nm nanodomain at the tips of stereocilia and this localization requires the presence of G\(\alpha_{i3}\), myosin 15 and whirlin. Using single-molecule tracking, we report that loss of Gpsm2 leads to decreased outgrowth and a disruption of actin dynamics in neuronal growth cones. Our results elucidate the aetiology of CMCS and highlight a new molecular role for Gpsm2/G\(\alpha_{i3}\) in the regulation of actin dynamics in epithelial and neuronal tissues.
Cdule-McCullough syndrome (CMCS, OMIM 604213) is a rare autosomal recessive neurological disorder in humans, characterized by early and severe onset of sensorineuronal deafness and hypoplasia of the corpus callosum (CC)\(^1\). CMCS syndrome patients often display frontal polymicrogyria (excessive small folds in the cortex of the brain) and heterotopia. These may be associated with cerebellar dysplasia, arachnoid cysts and ventriculomegaly. Some degree of delayed mental development has been reported for some patients, as well as occasional seizures, with overall psychomotor development generally normal\(^2\). The reason for this pleiotropy is not known and neither is the molecular basis of the pathology.

Recently, mutations in G-protein signalling modulator 2 (GPSM2 in humans, also known as Leu-Gly-Asn repeat-enriched protein (LGN), mammalian Partner of inscutable (mPins) or Gpsec2 in mammals) were found to cause CMCS\(^2\)–\(^4\). Because of postmitotic hair cells (HCs) of the mammalian cochlea\(^6,7\), a modulating actin dynamics. The versatility of Gpsm2/Gnai3 neurons from whirlin. Using live super-resolution imaging, we show that actin identified in CMCS patients affect protein complexes, including mutations cause of the early deafness and hearing deficits observed in deletion of either gene prevents stereocilia elongation, the likely result from defects in asymmetric division of progenitors, both in the inner ear and the brain\(^5\). Recently, we demonstrated that Gpsm2 and one of its binding partner, the \(z\)-subunit of the heterotrimeric G-protein \(G_{i3}\) (\(G_{i3}\) encoded by \(Gnai3\)), control the asymmetric localization of the kinocilium in developing postmitotic hair cells (HCs) of the mammalian cochlea\(^6,7\). These results were confirmed by another group that also reported an accumulation of both proteins at the tip of the hair bundles of the HCs\(^8,9\).

A HC stereocilia bundle is an actin-rich organelle consisting of a specialized array of microvilli-derived structures that protrude from the apex of auditory and vestibular HCs. Mechanical deflection of the hair bundle gates mechanosensitive ion channels in stereocilia that leads ultimately to afferent action potentials being conveyed to the central nervous system. Proper development and maintenance of stereocilia are vital for normal hearing\(^10\). Mechanisms that control HC bundle length are not fully understood. During early postnatal development, actin monomers are added at the barbed (plus) end of the stereocilium, resulting in elongation from the tip\(^11,13\). Several proteins are known to control this process\(^14,15\), with one common property being their localization at the tips of stereocilia during elongation\(^16\). The scaffold protein whirlin (encoded by \(Whrn\)) and myosin 15 (encoded by \(Myo15\)) are two of the best-characterized proteins among the HC bundle proteome participating in stereociliary growth. Myosin 15 interacts with whirlin and traffics it to the stereocilia tips where they colocalize with the actin-bundling and capping protein Eps8 (epidermal growth factor receptor pathway substrate 8)\(^17–19\), also dependant on myosin 15 for its localization at the stereociliary tip. Defects in all three genes individually cause abnormally short stereocilia\(^19–22\), deafness in mice\(^19,21,23\) and non-syndromic human deafness\(^24–26\).

In this study, we show that Gpsm2 and \(G_{i3}\) define an \(~200\) nm domain at the tip of stereocilia, and that conditional deletion of either gene prevents stereocilia elongation, the likely cause of the early deafness and hearing deficits observed in mutant mice. Furthermore, we demonstrate that mutations identified in CMCS patients affect protein complexes, including a novel and functionally relevant interaction between Gpsm2 and whirlin. Using live super-resolution imaging, we show that actin dynamics are disrupted in growth cones of young hippocampal neurons from Gpsm2 mutant mice, affecting neuronal outgrowth. These data support the idea of a global function for Gpsm2 in modulating actin dynamics. The versatility of Gpsm2/\(G_{i3}\) roles on actin and tubulin, in proliferative and postmitotic cells, is the probable cause of pleiotropy in CMCS brain anomalies.

**Results**

Gpsm2 and \(G_{i3}\) define a tip nanodomain within stereocilia. We evaluated the localization of Gpsm2 and \(G_{i3}\) during the development of stereocilia hair bundles using previously characterized specific antibodies\(^8\). Gpsm2 was localized at the tip of the nascent hair bundle at embryonic day 17.5 (E17.5), the earliest phase of its formation (Fig. 1a, yellow arrows). Consistent with previous observations, the apical crescent-shaped accumulation of Gpsm2 was also present (Fig. 1a, stars; refs 6,8). By postnatal day 7 (P7), when stereocilia are rapidly elongating, Gpsm2 and \(G_{i3}\) were enriched at the tips of the tallest row of inner hair cell (IHC) stereocilia, the actual sensory receptors receiving 95% of the fibres of the auditory nerve that project to the brain (Fig. 1b,ci), but also in vestibular HCs of the ampulla (Fig. 1d). At P15, the enrichment is maintained in the tallest row, whereas we could not detect fluorescence in the middle and small rows (Fig. 1e,f). At this stage, the apical crescent-shaped staining became fragmented or absent, suggesting a gradual loss of these proteins from this zone. Multicolour STimulated Emission Depletion (STED) microscopy was used to probe the stereocilia tip compartment and revealed that Gpsm2 was concentrated into a circular cap-like structure (Fig. 1f–i), similar to what was described for myosin 15 (refs 12,17,27), above the actin core labelled with phalloidin. Multicolour STED revealed that both Gpsm2 and Eps8 domains mostly overlapped (Fig. 1i). To evaluate the size of the tip domain, we mechanically isolated stereocilia after immunocytochemistry, to obtain perfectly flat structures (Fig. 1j,k). Using fluorescence intensity line-scans along individual long stereocilia labelled with Gpsm2 and Eps8 antibodies and using the full-width at half-maximum (FWHM), we estimated that the tip domain extended \(~200\) nm axially at the stereocilia tip (Gpsm2 FWHM = 198 ± 59 nm, \(n = 10\); Eps8 FWHM = 200 ± 63 nm, \(n = 10\)). These results reveal a narrow stereocilia tip compartment of \(~200\) nm where actin filament polymerization is regulated during hair bundle development.

**Loss of Gpsm2 or \(G_{i3}\) blocks stereocilia elongation.** Owing to their localization at the site of actin polymerization, we hypothesized that Gpsm2 and \(G_{i3}\) might be molecular components required for the developmental elongation of stereocilia. To test this, we examined the cochlear sensory epithelium of conditional mutant mice generated with a Foxg1-Cre driver (hereafter, named Gpsm2 cKO and \(G_{i3}\) cKO), during critical stages of stereocilia elongation. At P5, in early phases of elongation, scanning electron microscopy (SEM) analyses revealed an \(~40\%\) decrease in IHC tallest stereocilia length in Gpsm2 cKOs (see Methods) and \(~25\%\) decrease in \(G_{i3}\) cKOs, compared with controls (Fig. 2a–d). There was also a statistically significant increase in the number of stereocilia per bundle in IHCs of Gpsm2 cKOs (by \(~25\%)\) and \(G_{i3}\) cKOs (by \(~15\%)\), with abnormal supernumerary (more than three) rows of short stereocilia compared with control (Fig. 2e). In IHCs of Gpsm2 cKOs, the tallest row of stereocilia was reduced by \(~70\%)\) and \(~50\%)\) in Gpsm2 and \(G_{i3}\) cKOs, respectively (Fig. 2f–i, Supplementary Fig. 1a). The phenotype was more severe in Gpsm2 cKOs that have short, thick stereocilia. In \(G_{i3}\) cKO, we sometimes observed normal size stereocilia among an overall shortened hair bundle (Fig. 2h, magenta brackets), notably...
in the midbasal and more immature apical regions of the cochlea (Supplementary Fig. 1a, right panel, green arrows). In both cKOs, we observed supernumerary stereocilia per bundle, with ~60% and 50% increase in Gpsm2 and Gna13 cKOs, respectively, as compared with controls (Fig. 2j). These phenotypes are similar to those reported for Myo15, Whrn and Eps8 mutants20–22,29. Our results show that IHC bundles in Gpsm2 and Gna13 mutants share similar phenotypes, with stereocilia elongation significantly affected at the onset of hair bundle formation and an overall more severe phenotype in Gpsm2 cKOs.

Owing to the well-documented role of Gpsm2 (Pins) on spindle orientation during asymmetric cell divisions, hearing loss in CMCS patients was proposed to result from a defect in planar cell polarity2–4. Once apical/basal polarity has been established in mice, formation of the stereocilia bundle initiates in postmitotic HCs in the basal part of the cochlea coil. The critical phase of stereocilia elongation required to form the mature hair bundle occurs mostly after birth (P0). To confirm a postmitotic role for Gpsm2 in hair bundle elongation, we generated Gpsm2 conditional mutants using the Pou4f3 promoter to drive Cre-mediated recombination in postmitotic HCs only30 (Supplementary Fig. 1b). Hair bundles in these mutants displayed supernumerary rows of shorter stereocilia at P8 (Fig. 2j), similar to the phenotype we observed with an early embryonic deletion of the gene. To confirm a postmitotic function of Gz1 in stereocilia elongation, we treated cochlear explants from newborn rats (P0/P1) for 8 days in vitro (DIV) with increasing concentrations of pertussis toxin (PTX), a pharmacological inhibitor of all three Gz protein isoforms (Supplementary Fig. 1c). After 8 DIV, we found that stereocilia elongate in control cultures, whereas in the

Figure 1 | Gpsm2 and Gz13 are dynamically expressed at the tips of stereocilia. (a–c) Surface view of whole mounts of rat cochlear sensory epithelium at E17.5 (a) and P7 (b,c) illustrating Gpsm2 (a, green) and Gz13 (c, green) labelling in the actin-rich hair bundle labelled by phalloidin (Ph, magenta). (a) At E17.5, Gpsm2 localizes at the tips of the onset of hair bundle growth (yellow arrows), but also in an asymmetrical crescent in a distal region of the apical membrane of HCs (green asterisks). (b,c) By P7, both proteins accumulate at tips of stereocilia (green), strongly in IHC (yellow arrows) and more weakly in OHC (green arrows). Arrow: inner hair cell (IHC). Bracket: outer hair cell. Scale bars (a–c), 4 μm. (d) Gpsm2 (green) is localized at tips of P8 stereocilia of rat vestibular HC bundles. Ph: phalloidin. Scale bar, 2 μm. (e) At P15, confocal imaging reveals the accumulation of Gz13 protein at the tip of individual stereocilium. Scale bar 2 μm. (f–h) At P15, STED super-resolution imaging of the Gpsm2-expression domain at an individual stereocilium tip (f). Gpsm2 accumulated at tips of IHC stereocilia (green), above the F-actin labelling (magenta). (f, right panel, g) Acquisition of single plane images in two perpendicular axis as illustrated on the schematic in h reveals the cap-like structure of the Gpsm2 nanodomain. Scale bars, 2 μm. (i) Triple STED labelling reveals two mostly overlapping nanodomains at stereocilia tips with Gpsm2 (green) and Eps8 (magenta) above the F-actin signal (Ph, white). Left images: individual channels for Gpsm2 (top) and Eps8 (middle). The bottom image illustrates the phalloidin channel (grey) with two-colour binary representation of Gpsm2 (green) and Eps8 (magenta), with the overlapping domain (plain white). Scale bar, 2 μm. (j) Isolated long (j) and short (j, inset) stereocilia illustrate the accumulation of the two proteins in the long stereocilium only. Scale bar, 1 μm. (k) Intensity profiles of phalloidin, Gpsm2 and Eps8 from (j) (orange line across tip domain). Immunostainings repeated more than six times.
presence of PTX, hair bundles of IHCs exhibited supernumerary rows of short stereocilia (Fig. 2l), similar to those observed for the \(Gnai3\) cKOs. In these PTX-treated samples, the average length of the tallest row of stereocilia was reduced by \(\sim 30\%\) when compared with controls (Fig. 2m).

**Loss of Gpsm2 or \(Gz3\) causes hearing and balance deficits.** We assessed the auditory function of Gpsm2 and Gnai3 cKOs by measuring the threshold of auditory brainstem responses (ABR) using click-tones and pure tone of frequencies from 1 to 45 kHz. Gpsm2 cKOs were profoundly deaf at 6 weeks of age (Fig. 3a),...
with ABR thresholds above 90 decibel sound pressure level at all frequencies, whereas *Gna13* cKOs displayed specifically high frequency hearing loss starting at 11.3 kHz at the same age (Fig. 3b). These results are consistent with the differences in the severity of the phenotype we observed morphologically, and demonstrate that *Gpsm2* and *Gna13* are essential for stereocilia maturation and hearing. Another *Gpsm2* cKO resulting in a truncated protein lacking the carboxy terminus was recently reported to be deaf\(^\text{31}\). Also, *Gpsm2*, but not *Gna13* cKOs, exhibited increased hyperactive behaviour (413%) from controls and circling behaviour (>130 rotations in cKO) (Fig. 3c–f). *Gpsm2* cKOs swam in tight circles and are more mobile than their control littermates in a forced swim test (Fig. 3g). These results are indicative of a vestibular dysfunction and are consistent with the shortened stereocilia observed in the vestibular epithelium of the *Gpsm2* cKOs (Fig. 3h).

**Figure 3 | Gpsm2 and Gna13 mutations affect cochlear and vestibular function.** (a,b) Hearing tests on 4-week-old mice reveal severe threshold increases in *Gpsm2* cKO (a), compared with high frequency loss only in *Gna13* cKOs (b). Arrow in a indicates ABR thresholds exceeding the maximum testable intensity. Mean ± s.d. click-evoked ABR (click-ABR) and tone-burst-evoked ABR (f-ABR). Mean threshold values (in dB SPL) of click-ABR of control mice are shown above corresponding bars. **P<0.001 (Grey shaded area: P<0.05) by two-way ANOVA (post hoc Bonferroni’s multi comparisons test).** f-ABR: Control (Ctr) and cKO, n=8 ears from eight mice click-ABR: Control and cKOs: n=16 ears from eight mice. (c,d) Left panel: *Gpsm2* cKOs (green traces) display increased circling activity in a representative open-field during the first 30 s and at the end of the track (10 min) compared with control littermates, whereas *Gna13* cKOs (magenta traces) are unaffected (right panel). (e,f) *Gpsm2* cKOs mice cover more distance and rotate more than *Gna13* cKOs mice (each circle is an individual mouse). Open white circles are controls. (g) Top: heat map of force swim test occupancy for control and *Gpsm2* cKOs. Bottom: during the 2 min test, *Gpsm2* cKO mice showed less immobility and more body axis rotation compared with controls. (h) SEM of the surface view of the macula of the utricle of P11 mice in control (left) and *Gpsm2* cKO (right). Stereocilia elongation in the mutant is dramatically reduced compared with control. Scale bars, 1 μm.
Gpsm2/Gna13 need myosin 15/whirlin for stereocilia elongation. Since Gpsm2 and Gna13 mutants have short stereocilia similar to shaker 2 (sh2; myosin 15 functional null) and whirler (wi) mutants, we hypothesized that these proteins form a larger macromolecular complex. The delivery of whirlin to stereocilia tips requires functional myosin 15, whereas trafficking of myosin 15 can occur independently of whirlin. We found that Gpsm2 (Fig. 4a,b) and Gna13 (Fig. 4c,d) were both absent from the stereocilia tips of sh2/sh2 (Fig. 4a,c) and wi/wi HCs at P8 (Fig. 4b,d). These data demonstrate that Gpsm2 and Gna13 require myosin 15 to be trafficked to the tips of stereocilia. Myosin 15 was still present at the tip of short stereocilia of Gpsm2 and Gna13 cKOs at P8 (Fig. 4e,f), whereas whirlin localization was absent in Gpsm2 and Gna13 cKOs (Fig. 4g,h). Importantly, in earlier stages (P4) we observed that the apical crescent of Gpsm2 and Gna13 was maintained in sh2/sh2 mice (Fig. 5a,b). This result demonstrates that both proteins depend upon different interactions and protein complexes for apical membrane or stereocilia tip traffic within the HC. We also confirmed that the localization of both proteins at the tips of stereocilia was interdependent (Fig. 5c,d) as is the case.
in many other systems. Altogether, our data demonstrate that Gpsi2 and Gxα3 are sorted to the apical membrane and to the stereocilia tip via different protein interactions.

Our data suggest that whirlin is necessary for the trafficking and/or the maintenance of Gpsi2 and Gxα3 at the tip of the stereocilia, and absence of whirlin in Gpsi2/Gxα3 cKOs also suggest that the protein module participates in whirlin maintenance at the stereocilia tips. Altogether, our data show that the similar phenotypes of sh2, wi, Gpsi2, Gxα3 mutant mice arise from a common molecular function in driving stereocilia elongation.

**Gpsi2 and whirlin interact.** To further explore the functional interactions between myosin 15, whirlin, Gpsi2 and Gxα3 proteins, we used a heterologous system. COS-7 cells co-transfected with complementary DNA (cDNA) constructs encoding both myosin 15 and whirlin in numerous actin-rich filopodia protrusions and the two proteins accumulate at the tips of those extensions. Using this system, we show that Gpsi2 and Gxα3 were also transported to the tip of the filopodia in the presence of myosin 15 and whirlin (Fig. 6a,b). The majority of filopodia tips (99%) contained Gpsi2 in the presence of whirlin and myosin 15 and whirlin result in numerous actin-rich filopodia with complementary DNA (cDNA) constructs encoding both proteins, we used a heterologous system. COS-7 cells co-transfected with complementary DNA (cDNA) constructs encoding both myosin 15, whirlin, Gpsi2 and Gxα3. The majority of filopodia tips (99%) contained Gpsi2 in the presence of whirlin and myosin 15, and this number drops to 6% when an empty vector coding for DsRed replaces DsRed-whirlin (Fig. 6c). Co-immunoprecipitation experiments confirmed an interaction between full-length myc-tagged Gpsi2FL and either green fluorescent protein (GFP)–whirlin or untagged whirlin, whereas non-immune IgG or pEGFP–C3 coding for GFP did not co-immunoprecipitate either myc-Gpsi2 nor whirlin demonstrating the specificity of the interaction (Fig. 6d,e). To assess the interaction in the context of CMCS, we evaluated a human Gpsi2 variant reported in patients that is predicted to truncate the GoLoco and linker domains, and is associated with multiple and severe anatomical brain abnormalities. The Gpsi2R318RfsX8 variant still bound to whirlin, indicating that the N-terminal domain of Gpsi2 was sufficient for this interaction (Fig. 6d,f). We used glutathione S-transferase (GST)–pull down assays to show that the unstructured C-terminal region of whirlin, between aa 672 and aa 810 was the minimal domain required to interact with Gpsi2R318RfsX8 (Fig. 6d,g). All of the whirlin GST-constructs lacking this region failed to interact with myc-Gpsi2R318RfsX8 whereas the three GST-constructs containing this region pulled down the variant (Fig. 6g, lanes 4, 6, 8). To test if Gpsi2FL may act as an adapter stabilising whirlin at the tips of stereocilia, we transfected HEK293T cells with increasing amounts of myc-Gpsi2FL encoding cDNA while maintaining the quantity of DsRed-whirlin cDNA constant. Under these conditions, we observed a net increase in DsRed-whirlin protein expression levels, whereas increasing doses of DsRed-whirlin had no significant reciprocal effect on myc-Gpsi2 levels (Fig. 6h,i). Controls with increasing amount of cDNA encoding myc-Gpsi2FL did not affect DsRed levels (Supplementary Fig. 2c). One possible interpretation of these results is that when in a complex with Gpsi2, whirlin protein may be stabilized, possibly by being less susceptible to degradation.

As Gpsi2R318RfsX8 still bound whirlin, we evaluated if it could modulate the ability of myosin 15 and whirlin to generate filopodia. Figure 6d illustrates the position of Gpsi2 truncating variants identified in CMCS patients and the resulting predicted truncated proteins. In the presence of myc-Gpsi2FL, 42% of COS-7 cells extended filopodia, whereas in the presence of Gpsi2R318RfsX8, this number was reduced by half to 21% (Supplementary Fig. 2d, see Methods). These results suggest that the truncation of the linker and GoLoco domains in Gpsi2R318RfsX8 impairs the filopodia-generating ability of the myosin 15/whirlin complex. To evaluate if some of these truncation mutations could affect the proteins levels, we quantified immunoblots of the different Gpsi2 variants. Results show that the myc-Gpsi2FL variant (missing the last two GoLoco domains) had an ~15 ± 4% reduction in protein levels compared with the myc-Gpsi2FL, whereas myc-Gpsi2pG491GfsX6 variant (missing all four GoLoco domains) led to an ~70 ± 4%
Figure 6 | New protein complex between Gpsm2 and whirlin. (a–c) COS-7 cells transiently transfected with GFP–Myo15, DsRed–whirlin, myc–Gpsm2FL, untagged Gx3 or DsRed as indicated. (a) Co-expression of myosin 15 and whirlin leads to the formation of actin-rich filopodial structures with Gpsm2 accumulating at the tip with the two proteins. Absence of whirlin (DsRed only) markedly reduces the colocalization at the tips. (b) Gx3 also accumulates at the tip of filopodia with myosin 15 and whirlin. Transfections were repeated more than three times. Scale bars, 4 μm. (c) Percentage of filopodia tips (± s.e.m.) displaying colocalization of Gpsm2, myosin 15 and whirlin in the two contexts. n = number of filopodia tips from three independent experiments. ***P < 0.001 with Mann–Whitney test. (d) Schematic representation of Gpsm2FL and whirlin proteins. Four variants identified in CMCS patients, including Gpsm2R318RfsX8 are indicated. (e, f) Whirlin co-immunoprecipitates with myc-Gpsm2FL but not with non-immune IgG. (f) The interaction is maintained, though reduced, with the Gpsm2R318RfsX8 (Gpsm2R318R) variant. (g) A GST-pull-down assay indicates that the unstructured region of whirlin between aa 672 and aa 810 binds to Gpsm2R318RfsX8. Representative image of GST–whirlin pull-down assay. (h, i) Increasing amounts of myc-Gpsm2FL–encoding cDNA over constant amounts of whirlin leads to a net increase in whirlin expression levels, whereas increasing doses of whirlin had no significant effect on Gpsm2 levels. Five independent experiments presented as whisker box plots (min/max) combined with dot plot (blue bars representing the mean values) ***P < 0.001, **P < 0.01, *P < 0.05 (for h,i) with one simple t-test or one-way ANOVA (post-hoc Bonferroni’s test). NS, not significant. (j) Gpsm2FL significantly increased the length of filopodia generated by eGFP–myosin 15 and DsRed–whirlin (control) in COS-7 cells. Constructs bearing CMCS mutations led to shorter filopodia, compared with control or to Gpsm2FL. Data are presented as whisker box plots (min/max) from three independent experiments (n = 220, 207, 345, 228 and 188 filopodia, in the order indicated on the histogram’s x axis) (blue bars represent the mean values). ***P < 0.001, **P < 0.01 with Kruskal–Wallis test (post-hoc Dunn’s Multiple Comparison Test). NS, not significant.
decrease, and the Gpsm2R318RfsX8 variant (missing all four GoLoco domains and the linker domain) led to ~60 ± 4% decrease (Supplementary Fig. 2e). The shortest truncation (Gpsm2R127X), with only two tetratricopeptide repeats domains remaining, was the most severe (too weak for quantification). The impact of three of these four truncations was evaluated upon filopodia length in the presence of myosin 15 and whirlin. We first noted that co-expressing Gpsm2FL with myosin 15 and whirlin statistically increased the average length of filopodia (Fig. 6j). This increase was lost with all of the Gpsm2 variants tested. The truncated proteins also affected the myosin 15/whirlin effect, suggesting a dominant-negative effect. Notably, the shortest truncations retaining a amino-terminus (Gpsm2p.G491GfsX6, Gpsm2R318RfsX8) had the most severe effect on myosin 15/whirlin elongation. Taken together these data show that truncation mutations lead to some reduction in Gpsm2 protein levels, but they also indicate that the shortest forms still harbouring a N-terminus are able to interact with whirlin, therefore affecting the ability of myosin 15 and whirlin to induce filopodia elongation. We conclude that Gpsm2 is a new binding partner of whirlin, and that CMCS mutations affect the ability of the myosin 15/whirlin complex to generate filopodia.

Gpsm2 mutation affects neuronal development and motility. In addition to early-onset sensorineural deafness, patients with CMCS also display specific brain malformations on magnetic resonance images; hypoplasia of the CC being a hallmark of this pathology. To evaluate the impact of Gpsm2 on CC development, we generated Enx1-Cre+Gpsm2 cKOs (Gpsm2Emx1), deleting Gpsm2 in the early and dorsal telencephalon. Analysis of brains from these cKOs confirmed the existence of a caudal CC agenesis (Fig. 7a), as reported in CMCS patients. CC defects can result from a disruption in neuronal progenitors or defects in axonal elongation and guidance, which are dependent upon the microtubule and actin cytoskeleton. Neurite elongation is due to the motility of the growth cone driven in part by protrusive forces generated by actin polymerization at the leading edge, and the existence of a retrograde flow of actin resulting from a balance of filament polymerization and depolymerization, among other factors.

We therefore hypothesized that actin dynamics could be impaired in mutant Gpsm2 neurons and affect outgrowth. Measures of growth cone locomotion on N-cadherin-coated substrates showed that outgrowth of Gpsm2 cKOs neurons was reduced by 37% compared with controls (control 0.97 ± 0.04 µm min−1 versus cKO 0.61 ± 0.02 µm min−1), whereas the speed of growth cones from Gna13 cKOs was indistinguishable from controls (control 0.92 ± 0.02 µm min−1; cKO 0.89 ± 0.02 µm min−1) (Fig. 7b). This Gpsm2 cKO phenotype was also observed on a laminin-coated substrate, suggesting that the outgrowth reduction was not specific to an N-cadherin substrate (Fig. 7c). The number of pauses the growth cone made during the 30 min time-lapses on N-cadherin substrate was increased in Gpsm2 cKO neurons (Fig. 7d–f, Supplementary Movie 1).

To evaluate whether Gpsm2 regulates actin cytoskeleton dynamics we monitored the behaviour of individual actin-mEOS2 molecules using Single Particle Tracking combined with Photo-Activation Localization Microscopy (sptPALM) under Total Internal Reflection Fluorescence (TIRF) illumination, in the peripheral region of the growth cones (see Methods, Fig. 8a,b). We recorded trajectories longer than seven frames (median of nine frames, Fig. 8c), and fit the mean squared displacement (MSD) using a power function of time with exponent α (values between 0 and 2). This parameter reflects the curvature of the MSD function and the type of movement of actin-mEOS2 molecules, with α values close to 2 representing more directed trajectories, and α values close to 0 representing more static molecules (illustrated Fig. 8d). Comparisons analysis of the distribution of exponent alpha for control and Gpsm2 cKO shows statistical differences in the most extreme α values (below 0.6 and above 1.4; Fig. 8e). This demonstrates that actin-mEOS2 exhibit different dynamical behaviour in the peripheral region of growth cones of control and Gpsm2 cKOs. These results, together with the reduced neuronal outgrowth in Gpsm2 cKO, suggest that Gpsm2 affects motility of the growth cone through a modulation of actin dynamics.

The retrograde flow of actin is a complex phenomenon that is the result of many individual mechanisms, including actin filament nucleation and polymerization, capping and depolymerization, in addition to mechanical forces experienced from myosin contractility and coupling to adhesion molecules. Although we do not know the molecular complex that could drive Gpsm2 control of growth cone motility, we were able to co-immunoprecipitate (co-IP) endogenous whirlin with Gpsm2 from hippocampal lysates (Fig. 8f). We also found that co-expression of myc-Gpsm2 with DsRed-whirlin and GFP-myosin 15 in young (DIV3) hippocampal neurons lead to a striking colocalization of the three proteins at the tips of filopodia, supporting a role for these proteins in young neurons motility (Fig. 8g). To test if Gpsm2 could modulate actin polymerization, we compared the ratio of F-actin with G-actin (F/G) in cells transfected with both Gpsm2 and Gna13 constructs. When co-expressed, the proteins significantly increased the F/G-actin ratio by 138% (2.4 ± 0.2) compared with the control, demonstrating its impact on actin polymerization (Fig. 8h). The Gpsm2R318RfsX8 variant of the protein resulted in a statistically significant reduction of this activation (1.6 ± 0.1). A similar assay performed on cultured cortical neurons (see Methods), resulted in a 32% decrease in F/G-actin ratio in Gpsm2 cKO neurons compared with controls (0.68 ± 0.05) (Fig. 8i). These data support the hypothesis that Gpsm2 (with Gna13) stimulates actin dynamics in neurons, and that some of the brain anomalies observed in patients, notably CC hypoplasia, could be related to a disruption of Gpsm2-dependant actin-based mechanisms.

Discussion
In this study we show that the Gpsm2/Gna13 module regulates actin polymerization during stereocilia elongation, and that a pathogenic mutation of either gene leads to abnormally short stereocilia, the likely cause of hearing loss in CMCS patients. We demonstrate that this function is due to a newly identified interaction between Gpsm2 and whirlin, a member of the stereocilia tip complex. Also, we show that a Gpsm2 mutation affects CC formation and modulates neuronal outgrowth via the regulation of actin dynamics, supporting a global role for Gpsm2 in controlling the actin cytoskeleton.

Myosin 15 is the molecular motor responsible for delivering whirlin and Eps8 to the tips of actin-rich stereocilia and this ternary complex is required for elongation of nascent stereocilia. Our results show that myosin 15 and whirlin are also required for trafficking Gpsm2 and Gna13, two new members of the stereocilia tip complex, an electron-dense structure believed to contain proteins that regulate actin polymerization (Fig. 9). Accordingly, when myosin 15 is non-functional, Gpsm2 and Gna13 (this study), as well as whirlin17 and Eps8 (refs 19,22) are all absent from the stereocilia tips. On the other hand, when Whrn, Gpsm2 or Gna13 are mutated, myosin 15 still accumulates at the tips of short abnormal stereocilia. The failure of Whrn, Gpsm2 and Gna13 cKOs stereocilia to elongate despite the localization of myosin 15, suggests that these proteins assemble in a macromolecular...
complex (including Eps8) to regulate actin polymerization at the stereocilia tips. Interestingly, it was recently shown that the distribution of whirlin on the tallest row of stereocilia was dependent upon the isoform 2 of myosin 15 (short form)\(^3\). It is therefore probable that Gpsm2 and Gz\(_{13}\) are part of a preferential complex with myosin 15 isoform 2 and whirlin.

Our data extend the complexity of the interactions occurring at stereocilia tips during differentiation of HCs and provide new perspectives for the molecular machinery controlling actin polymerization. This complexity is highlighted by the increasing number of proteins identified and accumulating at the tip complex in a domain of \(\approx 200\) nm or less during stereocilia elongation (Fig. 9). In many cellular contexts, Gpsm2 and Gz\(_{13}\) are at the interface between actin and the membrane, acting as a link between the two\(^{3,5,10}\). In stereocilia, Gz\(_{13}\)-GDP could be tethered at the plasma membrane via its myristoyl and palmitoyl moieties, and bound to the C-terminal GoLoco motifs of Gpsm2. This would leave the tetratricopeptide repeats and linker region of Gpsm2 free to bind to various proteins (such as whirlin) to anchor or stabilize them, that could in turn interact with actin or other actin-regulatory proteins\(^{41}\), including Eps8 (refs 19, 22).

Our results significantly extend a recent report from Tarchini \textit{et al.}\(^3\), showing the localization of Gpsm2 (LGN) and Gz\(_i\) protein at the tips of P7 mouse HCs stereocilia, and reporting deafness and shortened stereocilia in a Gpsm2 mutant similar to ours. Importantly, we show that although myosin 15 is not required for the localization of Gpsm2 to the apical membrane crescent reported previously\(^3\), it is critical for the targeting of Gpsm2/Gz\(_{13}\) to the stereocilia tip. This demonstrates that Gpsm2 binds with different protein complexes to engage in distinct molecular mechanisms at these locations.

Based on our data, the early deafness observed in Gpsm2 cKO would be a consequence of a lack of postnatal elongation of stereocilia in IHC, the auditory sensory cells that are responsible for signal transduction, and which receive the vast majority of afferent innervation. Our results also uncovered Gz\(_{13}\) as a specific molecular partner for Gpsm2 during stereocilia elongation, notably within the basolateral cochlear region, and as a candidate gene for early-onset progressive hereditary hearing loss. Some of the differences in hearing loss (earlier and comprising the entire tonotopic axis in Gpsm2 mutants) probably reflect compensatory mechanisms by other Gz\(_i\) isoforms\(^2\).

Our data support the hypothesis that a decrease in actin polymerization, maybe through a disruption of the actin retrograde flow, underlies the reduced motility of Gpsm2 cKO neurons. In young postmitotic neurons there is an obvious molecular similarity between the mechanism controlling the elongation of the stereocilia, and those controlling the movements of the growth cone, including the existence of a filopodial tip.

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**Figure 7** | Gpsm2 mutation leads to hypoplasia of the corpus callosum and affects growth cone outgrowth. (a) Hematoxylin staining of coronal sections from P6 Gpsm2\(^{Emx1}\) control (upper panel) and cKO brains (lower panel) at a caudal levels. Note the absence of corpus callosum (CC) in the cKO mouse (inset). Scale bar, 1 mm, \(n = 4\) independent experiments. (b, c) In cultured hippocampal neurons at DIV2, N-cadherin-dependent outgrowth was reduced in Gpsm2 cKOs, but not in Gna13 cKOs (b). The reduction in outgrowth was maintained on a laminin substrate in Gpsm2 cKOs (c). Data from three to six independent experiments are presented as whisker box plots (min/max) (\(n = \) number of growth cones). ***\(P < 0.001\) with unpaired Student’s t-test. (d) Images from three time points (0, 15 and 30 min) of a time-lapse movie from 2 DIV control and Gpsm2 cKO hippocampal neurons plated on N-cadherin-coated glass and showing the difference in distance covered (dotted lines and double-headed arrows). Scale bar = 10 \(\mu\)m. (e) Quantification of the average number of pauses (\(\pm\) s.e.m.) during a 30 min period of growth cones from control (\(n = 31\) neurons) compared with Gpsm2 cKO (\(n = 51\) neurons), from four independent experiments. The Gpsm2 cKO growth cones pause more than controls. ***\(P < 0.001\), *\(P < 0.05\) with unpaired Student’s t-test. (f) Cumulated distance covered by control and Gpsm2 cKO growth cones over a 30 min period. Some pauses are indicated with blue arrows.
complex \(^{43-45}\) (Fig. 9). In both cases, it is the dynamic insertion of globular actin (G-actin) at the extremity of the structure (stereocilium or filopodium and lamellipodium) that allows elongation. This could be due to a role of Gpsm2 modulating the stability of the tip complex components, as we suggest for stereocilia elongation; a hypothesis supported by the endogenous co-IP of the Gpsm2 and whirlin from young hippocampi. All of the proteins identified so far that participate in stereocilia elongation are also expressed in the brain of mammals, including Gpsm2, Gα\(_{i3}\), myosin 15, whirlin and Eps8 (refs 46–50) (this manuscript). Myosin 15 is related to unconventional myosin 10 (MYO10), a powerful inducer of filopodia formation and elongation in neurons and other cells. Myosin 10 is present at the filopodia tip in bright puncta and remains there as the...
filopodia extend and retract. We find myosin 15 enriched in neuronal growth cones and most filopodia, and accumulating with whirlin and Gpsm2 at filopodia tips when co-transfected into hippocampal neurons. Myosin 15 could have overlapping functions with myosin 10 in neuronal protrusions, through a protein complex similar to the tip complex identified in the inner ear. But it is also possible that other neuron-specific binding partners for Gpsm2 participate in this process in neurons. Further studies are required to explore the exact molecular mechanism underlying this result.

Previous work with a Gpsm2 mouse model resulting in a truncated protein lacking the C-terminus suggested that cortical malformations in CMCS are due to abnormally localized apical progenitors, with no impact on neuronal production or forebrain thickness. The authors however did not report on the morphology of the CC. In our Gpsm2 cKO, we confirmed an overall normal cortical development and thickness, but we observed a severe hypoplasia of the CC, which appears to phenocopy a short and thin CC reported in the Palestinian patient carrying the p.R127X mutation in GPSM2 (refs 2,3). Our results demonstrating reduced neuronal outgrowth in Gpsm2 cKO neurons offer a mechanistic explanation for CC hypoplasia. The diverse anomalies observed in CMCS patients can be understood in the context of three cellular phenomena: (1) the

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**Figure 9 | Gpsm2 mutations affect stereocilia elongation and neuronal outgrowth by regulating actin dynamics at tip complexes.** Mechanistic model for Gpsm2-dependent stereocilia elongation and neuronal outgrowth. Gpsm2 accumulates at the tip complex of both structures via the myosin 15 motor protein in stereocilia and a comparable motor protein in filopodia. Gpsm2-dependent macromolecular protein complexes modulate actin dynamics at the tip of stereocilia (growing end) or the leading edge of the growth cone, participating respectively in the elongation and motility of the two structures. See text for details.

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**Figure 8 | Gpsm2 co-immunoprecipitates whirin in brain lysates and increases actin polymerization.** (a, b) Representative DIV2 growth cones from a control (a) and a Gpsm2 cKO (b) with the outlined peripheral region where sptPALM data were collected (yellow) and the corresponding individual actin-mEOS2 trajectories (3 min recording). Note the overall more-confined behaviour of the actin molecules in the cKO. Scale bars, 5 μm. (c) Distribution of the actin-mEOS2 trajectory length shows a median trajectory length of nine frames for both genotypes (blue line). (d) Representative mean squared displacement (MSD) over time for each of the three types of actin-mEOS2 behaviours with their corresponding a values. The plain curves represent fits to the function MSD = 4Dt, where D is a diffusion coefficient and z is a power law exponent. (e) Repartition of the z values of actin-mEOS2 molecules in control and Gpsm2 cKO neurons on N-cadherin substrate. Values from 11 (control) and 12 (cKO) growth cones from three separate experiments (+ s.e.m., n = 1344 trajectories for control and 1121 for mutant). ***P < 0.001, **P < 0.01, *P < 0.05 with an unpaired Student’s t-test or Mann Whitney test when a normality test failed. NS, not significant. (f) Immunoprecipitation of Gpsm2 together with whirin using anti-Gpsm2 serum. Membranes were immunoblotted with the antibodies indicated on the left. The experiment was replicated twice. (g) DIV3 hippocampal neurons electroporated with eGFP-myosin 15, DsRed-whirin and myc-Gpsm2 show enrichment of all three proteins at the tips of filopodia (arrowheads). Filopodia are outlined with dotted lines. The LUT was modified (left, Orange hot) to better visualise the accumulation. n = 5 independent experiments. Scale bar, 4 μm. (h) Actin assay shows that the combination of Gpsm2R181 and Gx13 expression shift the F/G-actin ratio, whereas the Gpsm2R318RfsX8 mutation decreases this shift. Dot plot from five biological repeats (black bar represent mean values). **P < 0.01 with Unpaired Student’s t-test, **P < 0.01 with one sample t-test. (i) Actin assays on cultures of neurons show a shift in the F/G-actin ratio, suggesting a decrease in actin polymerization in the Gpsm2 cKOs. Dot plot from five biological repeats (black bar represent mean values). **P < 0.01 with one sample t-test.
production and stability of the predicted truncated proteins, (2) the loss of GoLoco domains that are required for interaction with Gαi3, and (3) the loss or maintenance of domains that can impact on other binding partners of Gpsm2. This last point is illustrated here with results from the Gpsm2G491G63X and Gpsm2G1398RfsX8 variants whose expression leads to the production of truncated proteins with an almost complete N-terminus that is still able to bind to whirin and affect the ability of myosin 15/whirin to generate filipodia. Notably, compared with the shortest Gpsm2G392X variant5, the Gpsm2G1398RfsX8 variant is associated with some of the most severe brain anomalies described so far, perhaps because the level of this truncated protein is barely detectable.

Recently, we showed that Gpsm2/Gαi3 regulates early planar cell polarity in inner ear epithelia by modulating tubulin dynamics in postmitotic cells in a mechanism reminiscent of those controlling oriented cell division6. Here, we show that the same complex controls actin dynamics not only in postmitotic HCs but also in neurons, through different binding partners. Because Gpsm2 has many interacting partners and is involved in microtubule and actin dynamics, in both dividing and postmitotic cells, each mutation identified in CMCS patients might affect a variety of mechanisms. The expression of Gpsm2 in both neurons and glia44 adds another level of complexity to this, as the role of the protein in the latter has not been assessed. On the other hand, in the inner ear the deficits are very similar regardless of the mutation, with early-onset deafness identified in all patients, highlighting the absolute necessity of an intact Gpsm2 protein for hearing.

Altogether our study strongly suggests that the aetiology of CMCS, notably its complexity and multi-syndromic aspect, is due to the multifunctional role of the Gpsm2/Gαi3 module on actin and tubulin dynamics, in proliferative and postmitotic cells. This new molecular role for Gpsm2/Gαi3 in the regulation of actin dynamics in epithelial and neuronal tissues show that this protein complex plays sequential and/or partially overlapping roles in mechanisms controlling the polarized growth of tissues. Taken together, our work emphasizes the importance of identifying all interacting partners of Gpsm2 and Gαi3 and the mechanisms associated with each interaction, in different (patho)physiological contexts.

Methods

Transgenic mice used in this study. All procedures involving animals were done in accordance to the European Communities Council Directives (2010/63/EU) and the French National Committee (2013-118) recommendations. The French ‘Ministère de l’Éducation Nationale, de l’Enseignement Supérieur et de la Recherche’ approved all experiments under the authorisation no. APAFiS#1460-201508303720985 after agreement from the ethical committee of the University of Bordeaux. Gpsm2flox/flox cKO was described previously6. Generation of conditional Gnas3 KO mice. Exon 6 of the Gnas3 gene was flanked by loxP-sequences, because production of truncated proteins with an almost complete N-terminus that is still able to bind to whirlin and affect the ability of myosin 15/whirin to generate filipodia. Notably, compared with the shortest Gpsm2G392X variant5, the Gpsm2G1398RfsX8 variant is associated with some of the most severe brain anomalies described so far, perhaps because the level of this truncated protein is barely detectable.

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Cochlear explants cultures and PTX treatment. For cochlear cultures, cochleae from P0/P1 rat are placed in culture and the next morning (12–16 h) PTX was added at 1–100 ng ml−1, using a stock solution (50 mg ml−1). E15.5 cochleae were processed in the same tube at P5. For later stages, they were processed in different tubes comparing staining between controls and knockout, the two cochleae were processed in the same tube at P5 and 12/16 h later in the stages, they were processed in different tubes as only a small piece can be dissected at this stage, which renders final identification difficult. The samples were placed in mounting medium (Prolong Gold antifade reagent, Life Technologies) and flattened with a glass coverslip under a microscope.

For imaging acquisition, we used a confocal/STED microscope (TCS SP8: Leica) with a module STED × 3. Imaging was done using a 2 step from 0.25 to 0.35 μm. STED microscopy were performed with an objective 100 × 1.4 numerical aperture oil immersion STED objective. We used Atto 488 Phalloidin (Sigma, #49409, 1600) with a depletion laser of 592 nm, goat anti-mouse Atto 647 N (Sigma, #50185, 1-3000) and goat anti-rabbit Alexa Fluor 594 with a depletion laser of 775 nm. Confocal images were processed in Velocity software (Perkin Elmer) and Adobe Photoshop or ImageJ.

SEM and measurements. The inner ear (cochlear and vestibular system) of mice aged P5 and P21 were harvested and immersed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.35, with 3 mM CaCl2 for 24 h or more. The tissues were postfixed in 1% OsO4 in the same buffer, dissected and double processed with thiocarbohydrazide followed by OSM (ref. 61) before dehydration through an alcohol series and critical point drying with CO2. After mounting on specimen support stubs, samples were sputter coated with platinum. Samples were examined with a JEOL 6700 F cold field emission scanning electron microscope operating at 3 or 5 kV. Measurements were made on the longest row of stereocilia closest to the lateral wall (for P21) or on the site of the kinocilium on 84 control stereocilia/16 HCs/2 mice and 75/24/2 (for P5 and P21), on Gpsm2 cKO 36/82 and 108/212 (for P5 and P21), and on Gnas3 cKO 95/18/2 and 60/14/2 (for P5 and P21) in at least four different cochleae. The quantification of the number of stereocilia were made on 23 control HC2 mice and 12/2 (for P5 and P21), on Gpsm2 cKO 16/2 and 23/2 (for P5 and P21) and on Gnas3 cKO 19/2 and 27/2 (for P5 and P21) in at least four different cochleae.

Images were collected from the basal or middle turn of the cochlea, defined as ~20/30% and 50/60% of the total length of the organ of Corti from the base. At each location, the hair bundles were viewed both from behind the longest row of stereocilia to view the height of the hair bundle as well as approximately perpendicular to the apical surface of the HC or toward the inner aspect of the bundle to examine its overall morphology and composition. To estimate bundle height, measurements were made from images at calibrated instrument magnifications of ×20,000 or occasionally ×10,000. Although we might have underestimated the actual stereocilia length, all efforts were taken to minimize the effect of the stereocilia taken from bundles at early E10.5 (ref. 59).
that the row of longest stereocilia was approximately perpendicular to the direction of view. Stereo-imaging was used in a few cases to gain an indication of possible errors in length measurements from 2D images. From the anaglyphs generated by a pair of images separated by 8 degrees of tilt, height measurements were obtained using analySIS software. These revealed little difference in the height measurement from that obtained from the 2D image of a stereocilium at close to perpendicular to its long axis. Measurements were made using ImageJ software.

**ABR and vestibular tests.** ABR is measured by averaging the evoked electrical response recorded via subcutaneous electrodes. ABR to click and pure tone stimuli were recorded in anaesthetized mice aged 6 weeks. All physiological recordings were performed under anaesthesia (75 mg kg⁻¹ ketamine hydrochloride, Ketavet, Pharmacia, Pitzmoor, Germany; 5 mg kg⁻¹ xylazine hydrochloride (Rompun 2%, Bayer Leverkusen, Germany), 0.2 mg kg⁻¹ 1-adropin (Atropinsulfat B Braun, Melsungen, Germany) in a soundproof chamber (IAC, Niederkrüchten, Germany). In short, ABR thresholds were determined with click (100 μs), and pure tone stimuli (2–45 kHz, 3 ms duration).

For vestibular test, Gpm2 and Gnas3 mRNA were used for expression in vivo by injecting 10 μg of mRNA into the lateral ventricle of the brain of cKO mice and their control littermates (6 weeks old) were housed in a controlled environment (20–23°C) with free access to food and water and maintained on a 12 h/12 h day/night cycle, light on at 7 am. Mice litters were housed in collective cage and behavioural experiments were performed between 1 and 5 pm. Open-field and Forced swim test activities were analyzed with Ethovision (Version 11.5, Noldus Technology, Wageningen, The Netherlands). In the open-field test, mice were placed in the arena (30 × 40 cm) and activity was recorded for a period of 10 min. The total distance travelled and the number of rotations were measured (every 360° turn is counted as one rotation). In the forced swim test, mice were placed in a cylinder (height, 30 cm; diameter, 20 cm) filled with water (25°C). The activity, the total immobility and the number of rotation was recorded for a 2 min period. Immobility is defined by movements inferior to 2 cm s⁻¹.

**Filopodia quantification in COS-7 cells.** The constructs used were as follows: untagged Gx₃ (Origene), p-myc-Gpms2[E80R,Y81R]₄₈, pEGFP-c-MycXVa, pDsRed-c-Whirlin and DsRed-empty (Clontech) or p-myc-empty. Site-directed mutagenesis was used to generate mutations R127X (Gpsm2R127X), Q562X (Gpsm2Q562X), G491FgsX6 (Gpsm2G491FgsX6), and R318RxsX8 (Gpsm2R318RxsX8) on Gpms2 (QuickChange, Stratagene). We used an anti-myc (Covance mAb, MMS-150P-200, 1:1,000), anti-Gx₃ (ref. 60 and Sigma G4040, 1:1,000), anti-GFP chicken (Abcam, pAb #ab19709, 1:5,000), anti-DSRed (Living color, mAb #632392, 1:500 and Living color, pAb #632496, 1:2,000), and Phalloidin conjugated with coumarin (Sigma #P2494, 1:500). Images were acquired using Zeiss Axovision 4.7 and processed through Photoshop. For long-term assays (48 h), we considered a COS-7 (ATCC-American Type Culture Collection) ‘filopodial cell’ a cell with at least five long filopodia. For short-term assays of filopodia length, we split the cells the after 48 h of transfection, and re-plated them on coverslips previously coated with 5 mg ml⁻¹ of poly-i-Lysine (PLL), and left them adhere for 2 h, before they are processed for immunocytochemistry. For the quantification of percentage of colocalization at the tip of filopodia, 500,000 COS-7 cells were transfected by nucleofection using an Amaxa nucleofector kit (Lonza). After 48 h, the cells were re-plated on coverslips previously coated with 1 μg ml⁻¹ of PLL, and processed for immunocytochemistry after a 2 h adhesion. Cell appearing unhealthy were excluded from the analysis. Quantifications were performed blinded to the experimental group using ImageJ.

**Co-IP.** For Co-IP, HEK293T (ATCC-American Type Culture Collection) were cultured on 10 cm dishes, transfected using polyethyleneimine and harvested after 48 h in cold PBS. We used myc-tagged Gpms2 constructs and/or GFP–whirlin (whirlin was subcloned into the mammalian expression vectors pEGFP-C1, pEGFP-C3. Extracts were solubilized with Triton-X-100 (0.5% SDS) before harvesting at DIV3. For neuron stimulation, we used 20 mM KCl at a final concentration of 1.5%. The mixture was gently shaken, incubated for 15 min on ice and centrifuged for 1 h at 180,000 × g. The supernatant was neutralized with 2% Triton X-100 and the supernatant was incubated for 3 h at 4°C on glutathione-sepharose 4B beads. The beads were washed three times with PBS pH 7.5 containing 0.1% Triton X-100 and re-suspended in TBS pH 7.5 with proteases for later use.

**Western blot and immunoblot.** HEK293T cells in six-well plates were transfected with 50, 100, 200 and 300 ng of myc-Gpms2 in the presence of a constant concentration of DrsRed-whirlin or DrsRed-empty vector (30 ng), or with stable levels of Gpms2 with increasing concentrations of whirlin. In each condition, the total plasmid concentration was balanced with a control plasmid to a total plasmid of 350 ng plasmid. After 48 h, the cells were collected using cold PBS, centrifuged and solubilized by incubation for a few minutes in a lysis buffer. The lysis was centrifuged for 30 min at 15,000 × g. The supernatant containing myc-Gpms2 [3R18E5X8] overexpressed in HEK293T. HEK293T are re-suspended and sonicated in Tris-HCl pH 7.5 containing 5 mM EDTA, 1 mM sodium fluoride, 1 mM sodium orthovanadate, proteases inhibitors and then solubilized 30 min with 1% Triton X-100 and 0.5% SDS. The lysate was centrifuged for 30 min at 150,000 × g. The supernatant containing myc-Gpms2 [3R18E5X8] was incubated at ON at 4°C with GST antibody or GST-whirlin fusion proteins. After four washes with buffer containing 0.1% Triton X-100, the bead pellets were re-suspended in SDS sample buffer and subjected to SDS–PAGE and immunoblotting. Interaction was evaluated with anti-myc antibody (Covance, mAb, 1:1,000). To evaluate GST amounts, the samples are loaded on SDS–PAGE and stained with Coomassie blue.

**Actin polymerization assays.** The amount of F-actin and G-actin was evaluated according to the Cytoskeleton Actin Polymerization Assay Kit (BK037, Cytoskeleton) protocol and as previously described. HEK293T were transfected with a combination of pK-RFP/pK-YFP (control), or myc-Gpms2/YFP-Gx₃ (ref. 48). 24 h after HEK293T transfection we reduced the levels of fetal bovine serum in the medium from 10% to 1%, and the next day, the cells were stimulated with fresh medium containing 10% fetal bovine serum for 30 min, then re-suspended in F-actin stabilization buffer with adenosine triphosphate (1 mM) and protease inhibitors.

**Histology.** For histology, brains were harvested and fixed in Bouin’s fixative (Electron Microscopy Sciences) ON, dehydrated in ethanol, paraffin-embedded, and coronal sections (20 μm) obtained, before being stained with hematoxylin.
and mounted with Entellan (Millipore). Brain sections were examined using Leica MZ-16 stereomicroscope using the NanoZoomer 2.0-HT slide scanner and analysed with the Hamamatsu NDP viewer software (Hamamatsu).

Neuronal cultures and transfections. Hippocampal neurons were dissociated from E18 rat embryos as described and electroporated with 2 μg of eGFP–MYO15a, myc-GPSm2FL and DiRRed-whirin using Amaxa nucleofector kit (Lonza). Approximately 500,000 cells were transfected via nucleofection plated on coverslips treated with 10 μg ml−1 of PLL. After 2 days, neurons were fixed for 10 min with 4% paraformaldehyde at RT, then pre-incubated 30 min at RT in permeabilization buffer (PBS, 10%, NGS, 0.1% triton). Cells were then incubated at RT for 1 h with chicken anti-GFP (Abcam, Pab #ab13970, 1:3,000), anti-myc (mAb, Covance, 1:1,000) and anti-Di-Red (Clontech, Pab #632496, 1:3,000).

Fluorescent images of the neurons were obtained using a confocal microscope (Leica SP8) and processed with Adobe Photoshop.

Videomicroscopy and stpPalm-TIRF. Regular 18-mm glass coverslips were incubated for 2 h at 37°C with 1 mg ml−1 of PLL in 0.1 M borate buffer (pH 8.5), rinsed with H2O, then incubated 2 h at 37°C with 4 μg per coverslip of goat anti-human Fc (Jackson ImmunoResearch) in 0.2 M boric acid (pH 8.5), before another incubation ON at 4°C with 0.2 μg per cover (stpPalm-TIRF) and 0.6 μg per coverslip (time-lapse) of N-cadherin-Fc. Before use, the coverslips were rinsed again with borate acid. For laminin, coverslips were coated with 1 mg ml−1 of PLL and then laminin (5 μg per cover).

For time-lapse experiments (growth cone outgrowth), hippocampal neurons from newborn (P0/1) pups were plated at a density of 50,000 cells per coverslip. Neurons were used between DIV 4 and DIV 7. Neurons were transfected with 700 ng per well of plasmid DNA. For laminin, coverslips were coated with 1 mg ml−1 of PLL and then laminin (5 μg per cover).

Curvature of the MSD function and the type of movement. For highly directed movements, adjustable coefficient and the exponent m of the power law MSD is close to 2 (ref. 64), while maintaining filopodial activity.

The stpPalm-TIRF experiments were done as described in ref. 63. In brief, coverslips were mounted in a chamber and placed on a Nikon Ti Eclipse inverted microscope (Nikon France S.A.S., Champigny-sur-Marne, France) equipped with a TIRF arm coupled to a fibre optic linked to a four-colour laser bumper (Roper Scientific). Images were acquired using an Apo TIRF 100×/1.49 objective and a Photometrics (Tucson, USA) driven by the MetaMorph software (Molecular Devices, Sunnyvale, USA). The multi-positions were done with a motorized stage Scan IM (Marzhauser, Wetzlar, Germany). Temperature was maintained at 37°C. The system was configured by the MetaMorph software. Quantification of neuron growth cone speed was performed using ImageJ software plugin ‘manual tracking’. The average speed (in μm min−1) was quantified as this distance divided by 30 min. A ‘pause’ is when no movement (within the positioning accuracy of the growth cone centroid) of the growth cone is observed over two consecutive time-lapse images (1 min image−1), while maintaining filopodial activity.

The systems were evaluated using custom-made algorithms written as a MetaMorph plug-in as described in ref. 63. Single-molecule localization was performed using a wavelet-based algorithm, and trajectories were computed using a simulated annealing algorithm. The trajectory duration, which corresponds to the time during which single GFP fluorophores emit light upon 561 nm laser illumination, follows an exponential distribution strongly shifted to short values. Only trajectories longer than seven frames in regions of interest were kept, which yielded a median of around nine frames. The proportion of trajectories with more than seven points is 80% in control and 78% in mutants.

The numbers of trajectories analysed were 1344 (controls) and (1121) mutants.

Statistical analyses were carried out using Prism statistical package (GraphPad). Normality of distribution and homogeneity of variance were validated and statistical significance between means was calculated using unpaired Student’s t-test or Mann–Whitney test when normality test failed. P < 0.05 was considered significant.

Data availability. All data are available from the authors.

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The original version of this Article contained an error in the spelling of the author Aysegul Gezer, which was incorrectly given as Aysegul Geyser. This has now been corrected in both the PDF and HTML versions of the Article.

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