Stereocilia-staircase spacing is influenced by myosin III motors and their cargos espin-1 and espin-like

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Hair cells tightly control the dimensions of their stereocilia, which are actin-rich protrusions with graded heights that mediate mechanotransduction in the inner ear. Two members of the myosin-III family, MYO3A and MYO3B, are thought to regulate stereocilia length by transporting cargos that control actin polymerization at stereocilia tips. We show that eliminating espin-1 (ESPN-1), an isoform of ESPN and a myosin-III cargo, dramatically alters the slope of the stereocilia staircase in a subset of hair cells. Furthermore, we show that espin-like (ESPNL), primarily present in developing stereocilia, is also a myosin-III cargo and is essential for normal hearing. ESPN-1 and ESPNL each bind MYO3A and MYO3B, but differentially influence how the two motors function. Consequently, functional properties of different motor-cargo combinations differentially affect molecular transport and the length of actin protrusions. This mechanism is used by hair cells to establish the required range of stereocilia lengths within a single cell.

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Deceased
**Results**

**Loss of staircase organization in Espn-1 null hair bundles.** To test whether transport of Espn-1 by myosin-III affects stereocilia length, we used conventional gene targeting at the Espn locus to generate a mouse line (Espn-1<sup>−/−</sup>) that lacks Espn-1, but expresses short Espn isoforms (Fig. 1a). Stereocilia in the organ of Corti of Espn-1<sup>−/−</sup> mice appeared normal, with the exception of transient elongated microvillar-like protrusions on the hair-cell surface, opposite to the hair bundle (Fig. 1g); these protrusions disappeared by P10 (Fig. 1h). Espn-1<sup>−/−</sup> mice had normal hearing (Supplementary Fig. 1).

By contrast, ablation of Espn-1 produced stereocilia length abnormalities in specific regions of the otolith organs, the utricle and saccule, of vestibular sensory epithelia. These organs are used for detection of linear acceleration in rodents and can be subdivided into striolar and extrastriolar regions (Supplementary Fig. 2). The striola, a region of reduced hair-cell density<sup>12</sup>, is specialized for detection of dynamic (phasic) stimuli; the extrastriolar regions, which include medial and lateral extrastriola, encode tonic stimuli.<sup>23</sup> While hair bundles in the striolar region of the utricle and the saccule had normal morphology in Espn-1<sup>−/−</sup> mice (Fig. 1b,c), extrastriolar hair cells had bundles with a striking reduction in the slope of their staircase (Fig. 1d,e,j), caused both by a shortening of the tallest row of stereocilia in the bundle and elongation of shorter rows. Stereocilia lengths measured from scanning electron microscopy (SEM) images showed that the length difference between tallest and shortest stereocilia in extrastriolar bundles decreased from 10.8 ± 0.9 μm (mean ± s.d.; n = 6 cells) in heterozygotes to 1.9 ± 0.6 μm (n = 4) in Espn-1<sup>−/−</sup> mice, a reduction of &gt; 80%. Moreover, the diameter of vestibular stereocilia in the Espn-1<sup>−/−</sup> mouse was significantly less than those of wild-type (WT) mice (Fig. 1k–m). Nevertheless, Espn-1<sup>−/−</sup> mice showed no overt vestibular abnormalities (Supplementary Fig. 1a–d) and MYO15A was targeted normally (Supplementary Fig. 1f,g).

Because MYO3B lacks the actin-binding tail homology domain 2 (THD2), it cannot travel to distal tips of actin protrusions without interaction with Espn-1 (ref. 14). Indeed, in Espn-1<sup>−/−</sup> mice, MYO3B was no longer detectable at stereocilia tips of extrastriolar hair cells (Fig. 1n,o). Thus, at least in extrastriolar hair cells, Espn-1 is required for targeting of MYO3B to stereocilia tips, as well as regulation of stereocilia diameter and staircase formation.

**ESPN and ESPNL are prominent in mouse utricle hair bundles.** Surprisingly, PB538, an antibody directed against the ankyrin-repeat domain (ARD) of ESPN-1 (ref. 13), exhibited irregular immunoreactivity towards stereocilia tips in Espn-1<sup>−/−</sup> hair bundles (Fig. 1p). To search for ESPN paralogs expressed in hair bundles, we used the twist-off method<sup>10,24</sup> to isolate bundles from developing (~P4) and young adult (~P23) mouse utricles.<sup>25</sup> We detected proteins with shotgun mass spectrometry and liquid chromatography-tandem mass spectrometry (MS/MS) using an Orbitrap mass spectrometer, calculating each protein’s approximate molar abundance using relative iBAQ values.<sup>10,25</sup> Figure 2a,b shows the abundance of proteins detected in both bundles and epithelium at the two time points; the slope of the connecting lines indicates the bundle-to-epithelium enrichment<sup>25</sup>.

The ESPN-1 paralog ESPNL was highly enriched in hair bundles; on a volcano plot, ESPNL was the most statistically significant P4-enriched protein in the entire data set (Fig. 2c), emphasizing its high concentration in young utricle bundles. The summed splice forms of ESPN were also highly enriched and together were more abundant than ESPNL (Fig. 2a,b). MYO3A and MYO3B were present at low levels, although total myosin-III went up approximately threefold from P4 to P23. Examining peptide intensity that distinguished the two myosin paralogs, we estimated that the fraction represented by MYO3A went from ~20% at P4 to below the limit of detection in this assay at P23.

For greater quantification accuracy, we used parallel reaction monitoring (PRM), a targeted proteomics modality<sup>26</sup>, to specifically assay ESPN, Espn-1 and ESPNL in utricle hair-bundle extracts using 2–3 peptides each (Fig. 2d–h). Accurate quantification was assured by using quantified, heavy-isotope-labelled peptides as standards for each assay; assays were also validated by matching MS2 spectra collected at peaks to the appropriate protein in the mouse Ensembl database. By targeted proteomics, Espn-1 accounted for &lt;2% of the total ESPN; by contrast, ESPNL was 10-fold more abundant at P4 than ESPN-1. While Espn-1 and total ESPN declined in utricle bundles by less than twofold between P4 and P23, ESPNL decreased sevenfold (Fig. 2h). MYO3B was roughly equal in concentration to ESPN-1 at P4 and P23, while MYO3A was considerably less abundant (only detected in one out of four biological replicates at P4, and none at P23).

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**Cellular active-rich protrusions enable many biological functions, including migration, exploration and response to external stimuli<sup>1,2</sup>. Such functional diversity is made possible by variations in the dimensions, dynamics and positioning of actin structures within cells. In the inner ear, sensory hair cells are initially decorated with microvilli; these are remodelled during development to generate rows of stereocilia, which have precisely graded heights and form the mechanically sensitive hair bundle<sup>3</sup>. The bundle’s staircase organization is vital for its role in converting mechanical stimuli like sound to neural signals. Remarkably, stereocilia within a bundle can range from less than 1 to over 100 μm long<sup>4</sup>. The formation and maintenance of such an extraordinary range of lengths must require localized regulatory mechanisms within each stereocilium, and modulation must occur differentially across adjacent rows.<sup>5–7</sup> Although mechanisms of stereocilia height regulation are largely unknown, the significance of the problem is highlighted by the large number of deaf mouse mutants with abnormal stereocilia morphology<sup>8</sup>.

The dimensions of the parallel actin filament bundles that make up the stereocilium core are regulated by actin-binding proteins<sup>9,10</sup>, as well as unconventional myosin motors and their cargos<sup>11–13</sup>. Complexes of either of two unconventional myosins, MYO3A and MYO3B, together with their actin-regulating cargo ESPN-1, are candidates for controlling stereocilia lengths, based on several lines of evidence. Each of the three proteins localizes to the distal tips of stereocilia, the sites of actin polymerization, in a length-dependent distribution<sup>13–18</sup>. Second, Espn-1 is transported by MYO3A or MYO3B to tips of filopodia in cultured cells, as well as to stereocilia tips, where motor and cargo synergistically elongate these actin structures.<sup>13,14</sup> Third, mutations in MYO3A have been linked to DFNB30, a late-onset, progressive hearing loss<sup>19</sup>, and mutations in the Espn-1 gene, which encodes ESPN-1 as well as shorter splice forms, are associated with hearing and vestibular abnormalities.<sup>20,21</sup> In this study, we investigated the roles of myosin-III paralogs and ESPN-1 in stereocilia formation using Espn-1/C0 mice. In addition, we identified a novel myosin-III cargo, espin-like (ESPNL), which appears early in bundle development and is essential for normal auditory function. We show that these two myosins and their two cargos form a system that influences the number and the length of actin protrusions, and is critical for the formation of the normal stereocilia-staircase structure.
Figure 1 | Altered length and width regulation in Espn-1-/- mice. (a) Targeting strategy. PGK-Neo cassette targeted exon 1, which includes the translation start for ESPN-1; translation start for short ESPN isoforms is in exon 6. (b,c) Phalloidin-labelled stereocilia in the stria of Espn-1-/- utricles, imaged with confocal fluorescence microscopy, appear normal. (d,e) Phalloidin-labelled stereocilia in an extrastriolar region of Espn-1-/- utricles are of nearly uniform lengths. (f) SEM image of WT cochlear outer cell hair bundles. (g) SEM of P4 Espn-1-/- outer hair cells shows elongated protrusions opposite the bundle (arrow). (h) Extraneous protrusions disappear from Espn-1-/- outer hair cells by P10. (i) SEM image of WT utricle extrastriolar bundles. (j) SEM of extrastriolar bundles of Espn-1-/- utricle; stereocilia are of nearly uniform length. (k,l) Transmission electron micrographs show that extrastriolar stereocilia of Espn-1-/- utricle (l) have a smaller diameter than those of WT utricles (k). (m) Diameter of utricle (UTR) and saccule (SAC) stereocilia. Mean ± s.d.; n = 43 (WT utricle), 163 (Espn-1-/- utricle), 134 (WT saccule) and 168 (Espn-1-/- saccule). Differences are significant at P<0.001 (Student's t-test). (n) MYO3B targets to stereocilia tips of utricle bundles of WT mice. (o) MYO3B no longer targets to tips of Espn-1-/- stereocilia. (p) PB538 anti-ESPN-1 antibody detects antigen at some stereocilia tips (asterisks). Elongated protrusions (arrow) are visible in inner hair cells. Scale bars (b,d,m-o), 5 µm; (c,e-j), 2 µm; and (k,l), 100 nm.
Quantification of these proteins in the whole utricle ignores regional variations in expression. Using immunofluorescence in utricular wholemounts, we found that the myosin-III paralogs and their esp-related cargos showed striking enrichment in either striola (ESPNL and MYO3A) or extrastriola (ESPN-1 and MYO3B) zones (Fig. 2i–m). Higher magnification images revealed the boundaries clearly (Supplementary Figs 3–7).

Localization of ESPNL in hair bundles. As previously seen\textsuperscript{13,21}, a pan-ESPN antibody labelled the entire length of auditory and vestibular stereocilia (Fig. 3a,b). The PB538 ESPN-1 antibody labelled stereocilia tips of WT utricles; the signal was more concentrated at tips of taller stereocilia than shorter ones\textsuperscript{13} (Fig. 3c,d). Although this antibody cross-reacts with ESPNL, its reactivity is stronger towards ESPN-1 (Supplementary Fig. 1i–k). An antibody specific to the C-terminus of ESPNL (ab170747; Supplementary Fig. 1j) labelled stereocilia tips in both vestibular and auditory organs (Figs 3e,f); ESPNL was most concentrated at the tips of second and shorter rows. In utero electroperoration of GFP-ESPNL (Fig. 3i) and biolistic transfection of mEmerald-ESPNL (Supplementary Fig. 8h,i) confirmed targeting of ESPNL to stereocilia tips, particularly those of the second and shorter rows. Interestingly, in both cochle and utricle, ESPNL levels were remarkably non-uniform between stereocilia, including adjacent ones of the same length (Fig. 3e–i and Supplementary Fig. 8h,i). In early postnatal cochlea, this variability was most prominent in row 2; structured illumination microscopy (SIM) indicated that row 1 had much smaller but nearly uniform levels of ESPNL (Fig. 3g,h). Here ESPNL was detected with BG35961, also directed against the C-terminus (Supplementary Fig. 9d). In the utricle, labelling of variable intensity was seen in short- and intermediate-length stereocilia (Fig. 3e).

In the utricle, at P0.5, ESPNL was present in all hair bundles, although levels were higher in the striola; ESPNL was nearly gone from extrastriolar bundles by P7.5 and was not detected there at P20.5, despite robust expression in striolar bundles (Supplementary Fig. 8e–g). This result was consistent with the mass spectrometry quantification. In the cochlea, expression of ESPNL was transient, with expression levels falling between P3 and P10 (Supplementary Fig. 8a–d). The ab170747 and BG35961 antibodies reported similar ESPNL distribution in cochlea and utricle.

ESPNL binds but does not crosslink actin. While ESPN-1 and ESPNL are only 26% identical, ESPNL contains a 27-residue region homologous to a region within the actin-binding module of MYO3A and MYO3B\textsuperscript{13,14}. The ARDs of ESPN-1 and ESPNL co-localized strongly with the actin filament network (Fig. 4c) but unlike ESPN-1 (Fig. 4b), did not induce the formation of actin bundles or protrusions. When co-expressed with known actin-crosslinking proteins PLS1 (ref. 27) and ESPN-3 (ref. 17), ESPNL targeted to bundled actin (Fig. 4d,e). ESPNL thus binds to but does not crosslink actin, presumably because it lacks the additional actin-binding modules present in ESPN-1.

High-frequency hearing loss in Espnl null mice. To determine the role of ESPNL in hair-bundle function, we generated a knockout mouse line using the clustered regularly interspersed short palindromic repeat (CRISPR)/Cas9 nuclease technique\textsuperscript{28}. By delivering guide RNAs (gRNAs) directed at exons 1 and 8, we generated a 25 kb deletion in the Espnl gene (Espnl\textsuperscript{D}; Fig. 4f, Supplementary Fig. 9a,b). Complete loss of antibody labelling with BG35961 in the cochlea (Fig. 4g,h) indicated that this mutation was a null. We identified ~15 additional alleles with insertions or deletions in either exons 1 or 8 (Supplementary Fig. 9c). We examined one of these (Espnl\textsuperscript{D}), which has the addition of a single adenine in exon 8 and is predicted to form a truncated protein. Indeed, no immunoreactivity was detected in mice homozygous for this mutation using the BG35961 antibody.

When assessed by confocal microscopy, the overall morphology of early postnatal hair bundles appeared normal in Espnl\textsuperscript{A/A} cochleas. When measured by auditory brainstem responses (ABR) at ~1 month, Espnl\textsuperscript{A/A} and Espnl\textsuperscript{D/D} mice had high-frequency hearing loss (Fig. 4i). The tm1b allele of Espnl produced and phenotyped by the International Mouse Phenotyping Consortium had a very similar high-frequency hearing loss (http://www.mousephenotype.org/data/genes/MGI:2685402). Examination of bundles of P10.5 cochlear outer hair cells in basal regions, where high frequencies are encoded, showed that the shortest row (row 3) of stereocilia was mostly missing from Espnl\textsuperscript{A/A} bundles (Fig. 4j,k). Outer hair cells from mid regions and the apex appeared normal, as did all inner hair cells. At P30.5, the pattern was similar, suggesting that there was no degeneration of hair cells lacking row 3 stereocilia. ESPNL was present at P9 stereocilia tips at similar levels in apical and basal hair bundles (Supplementary Fig. 10), suggesting that the ESPNL phenotype was independent of the presence of ESPN-1. The organization of hair bundles in Espnl\textsuperscript{A/A} utricles appeared relatively normal in both striolar and extrastriolar regions, and no overt vestibular phenotype was observed.

ESPNL is transported by MYO3A and MYO3B. The ESPN-1 ARD interacts with the tail homology domain 1 (THD1) of both MYO3A and MYO3B\textsuperscript{13,14}. The ARDs of ESPN-1 and ESPNL are 26% identical, ESPNL contains a 27-residue sequence in the middle of the protein that shares 74% sequence homology with a region within the actin-binding module of ESPN-1 (Fig. 4a). Indeed, when expressed in COS7 cells, tagged ESPNL co-localized strongly with the actin filament network (Fig. 4c) but unlike ESPN-1 (Fig. 4b), did not induce the formation of actin bundles or protrusions. When co-expressed with known actin-crosslinking proteins PLS1 (ref. 27) and ESPN-3 (ref. 17), ESPNL targeted to bundled actin (Fig. 4d,e). ESPNL thus binds to but does not crosslink actin, presumably because it lacks the additional actin-binding modules present in ESPN-1.

![Figure 2 | Proteomics of developing mouse hair bundles.](image-url)
share 54% sequence homology and are predicted to fold similarly (Fig. 4a; Supplementary Fig. 11). Using pull-down assays, we found that purified GST-tagged ESPNL-ARD bound to GFP-tagged MYO3A-THDI (Fig. 5b) or MYO3B-THDI fragments (Fig. 5c), but not to regions N- or C-terminal of the THD1. When expressed together in COS7 cells, ESPNL-ARD was transported with MYO3A to filopodia tips (Fig. 5d). By contrast, ESPNL-ARD and MYO3B remained diffuse in the cytoplasm (Fig. 5e). When
full-length ESPNL was co-expressed with either MYO3A or MYO3B; however, ESPNL co-localized with each motor at filopodia tips (see Fig. 7). Thus the ARDs of ESPN-1 and ESPNL each bind to the THD1 of the myosin-III paralogs, but like with ESPN-1 (ref. 14), other domains of ESPNL enable MYO3B tip-directed motility.

**Myosin III paralogs substitute for each other in hair cells.** Results seen with the Espn-1 and Espnl knockouts suggested that cargo interaction with each myosin-III paralog was important for hair-cell function. In the mouse utricle, MYO3A was elevated in hair cells of the striolar region (Fig. 2l), and concentrated at the distal tips of auditory (Fig. 6a–c) and vestibular (Fig. 6d) hair cells. This coincided with an increase in high-frequency hearing loss in 30-day Espnl null mice (Fig. 6g). Espnl null cochlear outer hair cells from 30-day null mice exhibited significant high-frequency hearing loss, as demonstrated by ABR analysis (Fig. 6f).

**Figure 3 | Immunolocalization of ESPN-1 and ESPNL.** (a,b) Pan-ESPN antibody (green) and actin (magenta) labels utricle (a) and cochlear outer hair cell (b) stereocilia throughout. (c,d) ESPN-1 antibody labels the tips of utricular (c) and cochlear (d) stereocilia. (e) ESPNL antibody (ab170747) labels some stereocilia tips strongly (arrow), some weakly (asterisk), but does not label tallest stereocilia of utricle hair cell. (f) ESPNL antibody labels most but not all stereocilia tips of row 2 in bundle of outer hair cell. (g) Structured illumination microscopic image of ESPNL labelling (BG35961) of inner hair-cell bundles. (h) Quantification of ESPNL labelling of one hair bundle from (G). (i) Yellow lines indicate quantification trajectories; and (right) uniform labelling in row 1 but saturation of some tip signals in row 2 (asterisks). (j) GFP-ESPNL labelling of P0.5 inner hair cells following in utero electroporation at E12. Scale bars, 2 μm.

**Figure 4 | ESPNL functional characterization.** (a) Domain structure of ESPNL and ESPN-1. Blue, ankyrin repeats (ANK); black, proline-rich domain (P); red, WH2 domain (W); green, common actin-binding region (C); yellow, ESPN actin-binding domain (AB); and orange, ESPN C-terminal domain (CTD). (b) COS7 cells transfected with mCherry-ESPN (green), also labelled with phalloidin (magenta). Individual channels shown below. ESPN-1 forms filopodia. (c) mCherry-ESPNL localizes with actin structures but does not induce filopodia. (d) ESPNL localizes to filopodia induced by the actin-bundling protein GFP-PLS1. (e) ESPNL localizes to filopodia induced by GFP-ESPN-3. (f) CRISPR targeting strategy. gRNA-1 targeted exon 1 and gRNA-2 targeted exon 8; one of 16 alleles was a deletion between those two exons, eliminating the cargo interaction with each myosin-III paralog important for hair-cell function. In the mouse utricle, MYO3A was elevated in hair cells of the striolar region (Fig. 2l), and concentrated at the distal tips of auditory (Fig. 6a–c) and vestibular (Fig. 6d)
stereocilia. Although enrichment of MYO3A was largely correlated with stereocilia length\(^1\), higher magnification imaging in utricle bundles showed that MYO3A was low in the very shortest stereocilia, rose in intensity in the intermediate-length stereocilia, and then fell off in intensity in the longest stereocilia (Fig. 6d). By contrast, MYO3B was concentrated in the longest stereocilia, although it was also present at lower levels at the tips of other stereocilia (Figs 1m,6e).

To examine the role of the myosin-III paralogs within stereocilia, we generated Myo3a\(^+/−\) and Myo3b\(^−/−\) mice (Fig. 6). Hair-bundle organization and structure in auditory organs of both mutant lines appeared to be normal by SEM and confocal fluorescence microscopy; moreover, hearing thresholds were normal, even at 13 months (Fig. 6i,k).

While neither Myo3a\(^−/−\) nor Myo3b\(^−/−\) mice showed any overt vestibular dysfunction, we did note a subtle deficit in stereocilia-staircase step spacing in the extrastriolar regions of the utricle. Blind measurements of hair bundles imaged with confocal microscopy showed that Myo3b\(^−/−\) extrastriolar hair cells had a significantly smaller length difference between tallest and shortest stereocilia than did Myo3a\(^−/−\) or WT mice (Fig. 6l–q). This effect was reminiscent of that seen with Espn-1\(^−/−\) mice, but in Myo3b\(^−/−\) hair cells was due mostly to net elongation of short stereocilia (Fig. 6o,p). Shortening of tall stereocilia in Myo3b\(^−/−\) hair cells, while statistically significant, was less noticeable.

We next attempted to generate homozygous null Myo3a\(^−/−\) and Myo3b\(^−/−\) mutants. We used several different breeding schemes to generate double-null mice (Supplementary Fig. 12). Although expecting ~12 double knockouts from our 88 progeny, we obtained none. We also obtained no Myo3a\(^+/−\) Myo3b\(^−/−\) mice, despite expecting ~9. Using a \(\chi^2\)-test and computing P values by Monte Carlo simulation\(^29\), the obtained genotype distribution differed from that expected by \(P<0.001\). Double-null embryos presumably died during development, and a single WT allelic of Myo3a was unable to substitute for the loss of both Myo3b copies.

Filopodia elongation depends on cargo and myosin III. COS7 cell filopodia provide a useful assay for the effects of the myosins and their cargos on actin-protrusion elongation. To determine how ESPN-1 and ESPNL interact with myosin-III paralogs, we expressed various combinations of tagged proteins in COS7 cells, and monitored filopodia initiation and length (Fig. 7). To simplify our analysis, we used myosin-III constructs that lacked the N-terminal kinase domain, which normally autoinhibits the myosin motor activity\(^30,31\). Consistent with previous reports\(^14\), MYO3A alone induced the formation of filopodia, while MYO3B did not (Fig. 7a). When co-expressed with ESPN-1, MYO3A and MYO3B each produced filopodia that were significantly longer than with the motor alone (Fig. 7p; Supplementary Table 1).

ESPNL had strikingly different effects on filopodia elongation depending on which myosin-III was co-expressed. Cells expressing MYO3A and low levels of ESPNL had filopodia with ESPNL at their tips (Fig. 7a,g); as the expression level of ESPNL increased; however, filopodia eventually disappeared (Fig. 7g) and MYO3A co-localized with actin structures in the cytoplasm (Fig. 7b). The number and mean length of filopodia in cells...
expressing MYO3A and ESPNL were inversely correlated with the ESPNL concentration (Fig. 7g,h). By contrast, in cells expressing MYO3B and ESPNL, co-transport of both proteins to filopodia tips was consistently observed in cells expressing either low or high levels of ESPNL (Fig. 7c,d). With MYO3B, ESPNL concentration and number or mean length of filopodia were uncorrelated (Fig. 7i,j). In both cases, the effects of ESPNL on filopodia number and length were highly significant (Supplementary Table 1).

When MYO3A was present with high ESPN-1 and low ESPNL within a filopodium, all three proteins moved together to the distal tip (Fig. 7nD). The fluorescence-intensity profile of ESPN-1 MYO3A Actin MYO3B Actin MYO3A GFP-ESPNL MYO3B GFP-ESPNL MYO3A Actin MYO3B Actin MYO3A GFP-ESPNL MYO3B GFP-ESPNL MYO3A Actin MYO3B Actin MYO3A GFP-ESPNL MYO3B GFP-ESPNL MYO3A Actin MYO3B Actin MYO3A GFP-ESPNL MYO3B GFP-ESPNL MYO3A Actin MYO3B Actin MYO3A GFP-ESPNL MYO3B GFP-ESPNL MYO3A Actin MYO3B Actin MYO3A GFP-ESPNL MYO3B GFP-ESPNL MYO3A Actin MYO3B Actin MYO3A GFP-ESPNL MYO3B GFP-ESPNL MYO3A Actin MYO3B Actin MYO3A GFP-ESPNL MYO3B GFP-ESPNL MYO3A Actin MYO3B Actin MYO3A GFP-ESPNL MYO3B GFP-ESPNL MYO3A Actin MYO3B Actin MYO3A GFP-ESPNL MYO3B GFP-ESPNL MYO3A Actin MYO3B Actin MYO3A GFP-ESPNL MYO3B GFP-ESPNL MYO3A Actin MYO3B Actin MYO3A GFP-ESPNL MYO3B GFP-ESPNL MYO3A Actin MYO3B Actin MYO3A GFP-ESPNL MYO3B GFP-ESPNL MYO3A Actin MYO3B Actin MYO3A GFP-ESPNL MYO3B GFP-ESPNL MYO3A Actin MYO3B Actin MYO3A GFP-ESPNL MYO3B GFP-ESPNL MYO3A Actin MYO3B Actin MYO3A GFP-ESPNL MYO3B GFP-ESPNL MYO3A Actin MYO3B Actin MYO3A GFP-ESPNL MYO3B GFP-ESPNL MYO3A Actin MYO3B Actin MYO3A GFP-ESPNL MYO3B GFP-ESPNL MYO3A Actin MYO3B Actin MYO3A GFP-ESPNL MYO3B GFP-ESPNL MYO3A Actin MYO3B Actin MYO3A GFP-ESPNL MYO3B GFP-ESPNL MYO3A Actin MYO3B Actin MYO3A GFP-ESPNL MYO3B GFP-ESPNL MYO3A Actin MYO3B Actin MYO3A GFP-ESPNL MYO3B GFP-ESPNL MYO3A Actin MYO3B Actin MYO3A GFP-ESPNL MYO3B GFP-ESPNL MYO3A Actin MYO3B Actin MYO3A GFP-ESPNL MYO3B GFP-ESPNL MYO3A Actin MYO3B Actin MYO3A GFP-ESPNL MYO3B GFP-ESPNL MYO3A Actin MYO3B Actin MYO3A GFP-ESPNL MYO3B GFP-ESPNL MYO3A Actin MYO3B Actin MYO3A GFP-ESPNL MYO3B GFP-ESPNL MYO3A Actin MYO3B Actin MYO3A GFP-ESPNL MYO3B GFP-ESPNL MYO3A Actin MYO3B Actin MYO3A GFP-ESPNL MYO3B GFP-ESPNL MYO3A Actin MYO3B Actin MYO3A GFP-ESPNL MYO3B GFP-ESPNL MYO3A Actin MYO3B Actin MYO3A GFP-ESP...
within the filopodium was shifted slightly upward relative to MYO3A, however, and ESPNL was shifted proximally (Fig. 7nD), suggesting that the two cargos compete to bind to MYO3A. By contrast, when both cargos were expressed with MYO3B, little ESPNL was near filopodia tips, unlike ESPNL-1 (Fig. 7nF).

MYO3B lacks the THDII domain of MYO3A (refs 14,32). When we co-transfected COS7 cells with ESPNL and a hybrid protein in which THDII from MYO3A was fused onto the C-terminus of MYO3B (MYO3B + THDII), the hybrid mimicked MYO3A. In cells expressing lower levels of ESPNL, filopodia were formed and both ESPNL and MYO3B + THDII localized to their tips; by contrast, in cells expressing high levels of ESPNL, formation and elongation of filopodia containing MYO3B + THDII was reduced or abolished (Supplementary Fig. 13). The actin-binding THDII domain thus causes MYO3A to respond to the level of ESPNL much differently than MYO3B.

The number and length of filopodia that were induced by ESPNL-1 and either MYO3A or MYO3B were affected differently when ESPNL was present. When high levels of ESPNL were co-expressed with MYO3A and ESPNL-1, both the number and length of filopodia formed were reduced. Instead, we observed increased formation of cytoplasmic actin cables along which ESPNL and MYO3A co-localized (Fig. 7e,o,p). By contrast, ESPNL enhanced filopodia elongation by ESPNL-1 and MYO3B, even at high ESPNL expression levels (Fig. 7i,o,p).

Filopodia formed in the presence of ESPNL-1 and both MYO3A and MYO3B were significantly longer than those formed in the presence of ESPNL-1 and only one of the myosin-III paralogs (Fig. 7l,p). ESPNL had a more modest enhancement of filopodia length in the presence of both motors, and ESPNL antagonized the lengthening seen with MYO3A, MYO3B and ESPNL-1 (Fig. 7m,p). When ESPNL-1 was co-expressed with MYO3A and MYO3B, ESPNL-1 and MYO3A co-localized at filopodia tips and the tip-to-base gradient of MYO3A was extended13 (Fig. 7nA, nC). MYO3B entered filopodia when ESPNL-1 was present, but was consistently proximal to MYO3A along the filopodial shaft with a very extended tip-to-base gradient (Fig. 7nA, nC).

When we co-expressed ESPNL with MYO3A and MYO3B (Fig. 7nE), the position of MYO3B became more tipward than with ESPNL-1. Although exhibiting an extended comet-tail distribution pattern, MYO3B often localized more distally relative to MYO3A, frequently concentrating at filopodia tips. Conversely, MYO3A was often reduced or absent from filopodia tips, and was often only detectable towards the base of the filopodia immediately contacting the cell body. This result was consistent with the inhibition of filopodial tip-directed motility of MYO3A by ESPNL (Fig. 7b). When both cargos were co-expressed with MYO3A and MYO3B (Fig. 7nb), we observed further variations in their relative distributions within filopodia. While MYO3B was still consistently distal along the filopodium, relative to MYO3A, small amounts of MYO3A sometimes accumulated at filopodia tips (Fig. 7nB).

**Discussion**

We show here that MYO3A and MYO3B, along with their actin-regulating cargos ESPNL-1 and ESPNL, form a cooperative system whereby different motor-cargo combinations control the number and length of actin protrusions produced by a cell. While MYO3A and MYO3B largely substitute for each other if two wild-type alleles of either motor are present, ESPNL-1 and ESPNL interact differently with the two motors and cannot replace each other fully, at least in hair cells. Most notably, the inner-ear phenotypes of *Espnl* and *Espn* null mice suggest that this motor-cargo system is used to control the step size of the stereocilia staircase.

We used filopodia formation in COS7 cells as a model system for understanding how cells control the number and length of cellular processes containing crosslinked parallel actin filaments, such as control of stereocilia length by hair cells. Acting within restricted compartments at distal tips, the myosin-III isoforms and their cargos control the length of actin-rich filopodia and molecular transport within them33. Expression in COS7 cells of MYO3A, MYO3B, ESPNL-1 and ESPNL alone and in combinations allowed us to work out the intricacies of their activities and interactions. As shown previously, MYO3A alone stimulated filopodia formation, but MYO3B did not13,14; when no motors were present, ESPNL-1 elongated filopodia but ESPNL did not. Combinations of motor and cargo revealed further complexities in the system. Most importantly, while ESPNL-1 and ESPNL could each combine with MYO3B to elongate actin processes, MYO3A-dependent elongation was activated by ESPNL-1 but inhibited by ESPNL. ESPNL also inhibited lengthening when expressed in combination with ESPNL-1, either with MYO3A alone or when both motors were present.

To probe the role of the myosin-cargo system in the inner ear, we analysed a series of knockout mouse lines. While MYO3A and MYO3B are differentially expressed in regions of vestibular organs, single *Myo3a* and *Myo3b* knockouts nevertheless showed that the two motors are almost interchangeable during stereocilia development. Moreover, subtle defects in stereocilia-staircase spacing of *Myo3b*−/− hair cells indicated that MYO3A cannot fully substitute for MYO3B, especially in regions like the extrastriolar utricle where little MYO3A is expressed. By contrast, there presumably was enough MYO3B in hair cells of *Myo3a*−/− mice to allow them to develop their stereocilia normally; indeed, the overall level of MYO3B in utricles is much greater than that of MYO3A, suggesting MYO3B is the predominant paralog. That said, humans who are homozygous or compound heterozygote for recessive *Myo3a* mutations suffer from non-syndromic hearing loss (DFNB30) that is progressive19, which suggests that

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**Figure 6 | MYO3A and MYO3B separately localize in bundles but replace each other in the ear.** (a) Immunolabelling of MYO3A (green) in rat auditory hair cells (P3); actin (magenta) labelled with phalloidin. (right) MYO3A label only. (b) MYO3A and ESPNL-GFP do not completely overlap in hair bundle of outer hair cell (see inset). Elongated ESPNL-GFP labelling in row 2. (c) SIM image of outer hair-cell stereocilia showing MYO3A cap and elongated ESPNL-GFP. (d) MYO3A labelling in utricle bundle. (left) MYO3A image with transect indicated. (right) Intensity profile along transect. (e) (left) MYO3A labelling is greatest in tallest stereocilia (asterisks). (right) Intensity profile along transect. (f) Representation of exons 1–7 (blue boxes) of WT mouse *Myo3a* locus. The targeting vector replaced exons 4 and 5 with a floxed neomycin-resistance gene cassette, introducing a premature stop codon in exon 6. (g) Exons 1–8 of the *Myo3b* locus; the targeting vector (middle) was designed to eliminate exons 3 and 4, replacing them with a neomycin-resistance gene cassette conjugated to LacZ; a premature stop codon was introduced in exon 5. (h) RT-PCR on inner-ear mRNA showing 578 bp amplicon from WT mouse and 448 bp fragment from *Myo3b*−/− mice. (i) Mean ABR measurements (± s.d.) from *Myo3a*−/− (n = 29) and *Myo3b*−/− (n = 27) mice at 6 months. (j) RT-PCR on inner-ear mRNA showing 695 bp amplicon from WT mouse and expected 319 bp truncated band from *Myo3b*−/−. (k) ABR from *Myo3b*−/− (n = 9) and *Myo3b*−/− (n = 9) mice at 6 months. (l) WT extrastriolar bundle (phalloidin stained). (m) *Myo3a*−/− extrastriolar bundle. (n) Myosin-III isoforms and their cargos control the length of actin-rich filopodia and molecular transport within them33. Expression in COS7 cells of MYO3A, MYO3B, ESPNL-1 and ESPNL alone and in combinations allowed us to work out the intricacies of their activities and interactions. As shown previously, MYO3A alone stimulated filopodia formation, but MYO3B did not13,14; when no motors were present, ESPNL-1 elongated filopodia but ESPNL did not. Combinations of motor and cargo revealed further complexities in the system. Most importantly, while ESPNL-1 and ESPNL could each combine with MYO3B to elongate actin processes, MYO3A-dependent elongation was activated by ESPNL-1 but inhibited by ESPNL. ESPNL also inhibited lengthening when expressed in combination with ESPNL-1, either with MYO3A alone or when both motors were present.

To explore the role of the myosin-cargo system in the inner ear, we analysed a series of knockout mouse lines. While MYO3A and MYO3B are differentially expressed in regions of vestibular organs, single *Myo3a* and *Myo3b* knockouts nevertheless showed that the two motors are almost interchangeable during stereocilia development. Moreover, subtle defects in stereocilia-staircase spacing of *Myo3b*−/− hair cells indicated that MYO3A cannot fully substitute for MYO3B, especially in regions like the extrastriolar utricle where little MYO3A is expressed. By contrast, there presumably was enough MYO3B in hair cells of *Myo3a*−/− mice to allow them to develop their stereocilia normally; indeed, the overall level of MYO3B in utricles is much greater than that of MYO3A, suggesting MYO3B is the predominant paralog. That said, humans who are homozygous or compound heterozygote for recessive *Myo3a* mutations suffer from non-syndromic hearing loss (DFNB30) that is progressive19, which suggests that
Figure 7 | ESPN-1 and ESPNL cooperate with MYO3A and MYO3B to differentially control filopodia growth. (a,b) COS7 cells co-expressing mCherry-MYO3A (magenta) with relatively low (a) or high (b) expression of mEmerald-tagged full-length ESPNL (green). (c,d) COS7 cells co-expressing mCherry-MYO3B (magenta) with relatively low (c) or high (d) expression of mEmerald-ESPNL (green). (e) MYO3A is unable to target filopodia tips in the presence of ESPNL, even when co-expressed with ESPN-1. (f) MYO3B consistently targets filopodia tips in cells expressing both ESPNL and untagged ESPN-1. (g–j) Quantification of relationship between ESPNL fluorescence level (ESPNL Fl) and filopodia density (g,i) or length (h,j). There was an inverse relationship of filopodial number and length with ESPNL fluorescence for MYO3A ($R^2 = 0.4$ and 0.5) but not MYO3B ($R^2 < 0.1$ and $< 0.01$). (k) COS7 cell expressing GFP-MYO3A (green) and mCherry-MYO3B (red), with actin labelled phalloidin (blue). (l) Filopodia are longer in the presence of MYO3A, GFP-MYO3B, TagBFP2-ESPNL (blue) and untagged ESPN1. (m) Confocal images and relative pixel intensity profiles of single filopodia from cells transfected with the indicated constructs. Colours are identical in images and corresponding profiles. Scale in the profiles applies to the images. (o,p) Filopodia number per 10 $\mu$m of cell perimeter (o) and lengths (p) for each motor-cargo combination are presented as box plots, with upper and lower whiskers representing the range, top and bottom of the boxes representing the upper and lower 25th percentile, and the bars bisecting the boxes representing the median values (values from $\sim 30$ COS7 cells used for each combination). Two-way analysis of variance analysis of data in these panels is reported in Supplementary Table 1. Scale bars, 2 $\mu$m.
substitution of MYO3B for MYO3A is not completely effective in the cochlea.

Double knockouts indicated at least one Myo3b allele is required for embryonic development; no Myo3a−/− Myo3b−/− or Myo3a−/− Myo3b−/− mice were born from crosses that should have generated substantial numbers of them. These results suggest that these motors are required in other tissues, in addition to inner ear14,15 and retina13, a prediction that is supported by examining their tissue expression using the Illumina Body Map (http://www.ebi.ac.uk/gxa/experiments/E-MTAB-513).

Myosin-III motors require ESPN-1 or ESPN to function in hair cells. ESPN was the most prominent developmentally regulated hair-bundle protein in our proteomics experiments, and was highly enriched in bundles. Like ESPN, ESPNL both binds to actin and has a proline-rich domain that may bind profilin-actin; this domain may allow ESPNL to concentrate actin monomers and contribute to stereocilia elongation16. Indeed, ESPNL allows filopodia to be formed with MYO3B. When low levels of ESPNL are present, MYO3A-dependent filopodia can be formed; by contrast, high levels of ESPNL inhibit filopodia elongation when MYO3A is present.

Like that of Espn-1−/− mice, the phenotype of Espnl−/− mice was apparent in only a subset of hair cells; while there were no apparent vestibular functional defects, sensitivity towards high-frequency auditory stimuli was drastically reduced. Consistent with high-frequency hearing loss, Espn−1−/− mice had altered bundles of outer hair cells—largely missing stereocilia row 3—only at the cochlea’s basal end, which encodes high frequencies. While the loss of one stereocilia row should decrease the total amount of transduction current by ~50%, auditory sensitivity may drop by far more than that given the special role of outer hair cells in cochlear amplification35. Given the greater ESPNL abundance in the second row, loss of the third row was puzzling: ESPNL activity in short stereocilia could, however, prevent stereocilia destabilization that is predicted to occur below a certain length37. Regardless, we presume that Espn−1−/− mice have a restricted phenotype because ESPN-1 can substitute in activating myosin-III molecules in most hair cells.

In COS7 cells, ESPN-1 stimulated filopodia initiation and lengthening regardless of whether MYO3A, MYO3B or both motors were present. In hair cells, ESPN-1 accounted for <2% of the total ESPN protein but was responsible for a key subset of functions carried out by ESPN. The morphology and function of hair bundles of Espn−1−/− mice differed from those of jerker mutant mice, which lack all ESPN isoforms and are profoundly deaf with severe vestibular dysfunction21,36,37. Homozygous jerker stereocilia completed neither diameter growth nor elongation during bundle formation, but instead bent, shortened and disappeared36. In contrast, stereocilia in auditory and vestibular hair cells of Espn−1−/− mice persisted through adulthood, albeit with a altered phenotype in extrastriolar hair cells.

While stereocilia diameter was reduced in extrastriolar hair cells of Espn−1−/− mice, consistent with the observation that ESPN-1 controls the size of actin bundles in vestibular hair cells31 or interaction with other cellular components49. Together this system allows the cell to precisely tune individual actin-regulatory cargos, while other Espn−1−/− hair cells presumably retained ESPNL-dependent activation of MYO3B. The more subtle defect in staircase formation observed in Myo3b−/− mice reinforced this interpretation; a small amount of MYO3A present in extrastriolar hair cells of Myo3b−/− mice presumably could partially control stereocilia spacing. By contrast, in other single knockouts we examined, when MYO3A or ESPNL were missing, each hair cell expressed detectable levels of both a myosin-III paralog and either ESPN-1 or ESPNL. For example, some hair cells of Espnl−/− mice still express ESPN-1, which can activate either MYO3A or MYO3B.

Although the myosin III-cargo system can lead to massive outgrowth of filopodia in COS7 cells, the Espn−1−/− extrastriolar phenotype showed that in hair cells, a critical function of this system is to instead control the relative step size of the stereocilia staircase. A comprehensive model of actin-process elongation explains the formation of staircase spacing by invoking gradients of actin-polymerization activators or physical properties at the apical surface8,7. Because multiple mechanisms control stereocilia length4,9,38–40, and the total actin content seems to be fixed31, the activity of the myosin III-cargo system must be proportionally constrained to differentially activate actin elongation in successive rows of stereocilia, so that the shortest rows have the least activation and the longest rows have the most. Indeed, the length dependence of MYO3A and MYO3B localization in stereocilia, as well as the phenotype seen in Myo3b−/− hair cells, both consistent with such a mechanism. Moreover, given the inhibitory activity of elevated levels of ESPNL on motor-cargo complexes that include MYO3A, the large amounts of ESPNL at tips of row 2 of cochlear inner hair cells may serve to disproportionately suppress growth of this stereocilia row, generating the much smaller rows 2 and 3 step spacing that of rows 1 and 2. Likewise, later in development ESPNL accumulates at the tips of row 3 stereocilia, presumably suppressing elongation there.

The functional significance of staircase step spacing is not fully understood. A small intrinsic gradation in stereocilia height seems to be an essential part of a bundle42, and may be set up at the earliest stage of bundle development43; a further increase in step spacing occurs after the minimal spacing is established35,36. While step spacing is thought to have little effect on hair-bundle stiffness44, this distance could control the diffusion of Ca2+ to the upper end of the tip link in vestibular hair cells and perhaps the rate of slow adaptation40,45.

The restricted phenotypes in Myo3a−/−, Myo3b−/−, Espn−1−/− and Espnl−/− mice reinforce the suggestion that robust compensatory mechanisms control stereocilia length. For example, ESPNL enhances outgrowth of MYO3B-dependent actin processes, yet inhibits those dependent on MYO3A or motor mixtures. When hair cells lack ESPN-1 or MYO3A, MYO3B-ESPNL complexes apparently rescue control of stereocilia length. Stereocilia must maintain the staircase for a lifetime, even though actin at tips is dynamic38,39,42,46–48, suggesting that considerable redundancy must be built in. Elimination by P10 of the ectopic long microvilli present in Espn−1−/− auditory hair cells may be an example of the hair cell’s ability to overcome transient misregulation of actin-protrusion length. The myosin-III plus cargo system operates using selective protein expression, targeting of individual motors to specific actin protrusions, strong stimulation of actin elongation by ESPN-1, and differential stimulation of elongation by ESPNL depending on motor expression; two motors and two cargos allows for combinatorial control. Endogenous regulation likely involves auto- and inter-molecular phosphorylation by the myosin-III kinase domains30,31 or interaction with other cellular components49.
actin protrusions' lengths, which is essential for the normal formation of elaborate cellular architecture like hair-cell stereocilia.

Methods

Animals. The care and use of animals for the experiments described conformed to NIH guidelines and were approved by the Institutional Animal Care and Use Committee of the National Eye Institute. The National Eye Institute on Deafness and Other Communication Disorders (NIDCD ACUC), Oregon Health and Science University (OHSU IACUC) and the University of Nebraska-Lincoln (UNL IACUC). In all experiments, a mixture of male and female mice were used. No gender-specific effects were noted.

Knockout mice. Standard methods were used to generate mouse founders carrying deletions of exon 4 and 5 in Myo3b allele, and of exon 1 in Espnl allele at the Animal Transgenic Facility of the National Eye Institute (Bethesda, MD). Exons 3 and 4 were targeted in Myo3b, which was carried out by the Texas A&M Institute for Genomic Medicine (College Station, TX). Final targeting vectors were trans-fected into mouse ES cells (SV129) by electroporation. After selection based on drug resistance, correctly targeted ES cell clones were screened by long-range PCR, verified by Southern blotting, and microinjected into blastocysts. At least five chimeric mice were crossed with C57Bl/6j partners for germline transmission of the modified allele. Founders for each genetically modified colony were selected by long-range PCR genotyping and were backcrossed onto the C57B6j background for five generations or more before phenotypic evaluation of homozygote mutants.

For the Espnl knockout with CRISPR, gRNAs targeted to Espnl exons 1 and 8 were selected using the CRISPR web based tool (http://crispr.mit.edu/). The gRNA sequences (exon 1, 5'-TGTCGATCATGCAGGAAAGGCT-3'; exon 8, 5'-CAGGGAGGGAGAAGGAGGGG-3') were individually cloned into the DR274 plasmid (Addgene #42227) and transcribed with the Megascript (LifeTechnologies). gRNAs were purified using the Nuclease spin mRNA (Machery-Nagel) and quantified. Zygotes were injected with a cocktail containing 30 ng/μl of each gRNA and 110 ng/μl of Cas9 mRNA (Trilink) and implanted into pseudopregnant females. All founders were screened for mutations in Espnl exon 1 and 8 and any intervening deletions between these two exons. We characterized two alleles in depth: c.135_1391del1165 (forming protein truncation at C-terminus, PCR-amplified Espnl was ligated into a similarly cut mCherry-N1 or mEmerald cloning vector to yield a fusion of ESPNL with mCherry or mEmerald, respectively. All DNA for transfection was prepared using the Plasmid Maxi kit (QIAGEN, Valencia, CA) and characterized by transfection in HeLa cells (CCL2 line; ATCC, Manassas, VA) using Effectene (QIAGEN) followed by observation under wide-field fluorescence illumination to ensure proper localization (ET-GFP and ET-Red filters; Chroma Technology, VT). The sequences for all vectors were confirmed using Big Dye technology by the Florida State University DNA Sequencing Laboratory in the Department of Biological Science. The GST-EPSNL construct was generated by fusing the GST tag to the N-terminus of ESPNL housed in a N1-Clontech vector. The following primers were used: to PCR amplify the GST tag and create Agell and BspEII restriction sites: Agell forward: 5'-GGGCTACGCTGCAACCTGTGGGGAATCTGAGTATTTGGAAAAATAAAGGCGCTTGTGGCACAC-3'; BspEII reverse: 5'-TCTGATTCCGGAGCAGGAACAGATCCGGATTGGAGATGTTC-3'. The PCR product was digested and ligated into a similarly cut ESPNL-N1 cloning vector to yield a fusion of GST with ESPNL separated by a 22 amino acid linker (GST-22-ESPNL).

Immunofluorescence and light microscopy. For experiments conducted in the NIDCD, mice were killed and temporal bones were dissected; they were then perfused through the round window with 4% formaldehyde in phosphate buffered saline (PBS; pH 7.4) and fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) for 24 hours. The specimens were micro-dissected in PBS before permeabilization with 0.5% Triton X-100 for 30 min, and blocking overnight at 4°C in filtered 4% bovine serum albumin (BSA) in PBS. In the case of PB791, tissue was treated for antigen retrieval before blocking, by incubating in 1 mTris-EDTA buffer pH 9 at 60°C for 1 h. To reduce non-specific binding for immunofluorescence assays, it was first incubated with a blocking antibody (1 μg ml⁻¹) for 2 h, rinsed with PBS three times, stained with goat anti-rabbit secondary antibodies (Molecular Probes) conjugated with AlexaFluor 488 (A-11001) or Alexa Fluor 568 (A-11011) diluted 1:600 in PBS-BSA for 1 h, and counterstained with AlexaFluor-568 phallidin (Molecular Probes, A-12379) or AlexaFluor-488 phallidin (Molecular Probes, A-22287) dissolved 1:600 in PBS-BSA. Tissue was then rinsed with PBS three times, and mounting used ProLong Gold Antifade reagent (Invitrogen). Confocal imaging of both tissue as well as cell-culture experiments was accomplished using a TIE inverted fluorescence microscope (Nikon Instruments) equipped with either (1) a spinning disk confocal head (Perkin-Elmer, DU-888 EMCCD) and Apo TIRF 1.49 NA objective, or (2) a swept-field confocal scan head (Prairie Technologies, DU-897 EMCCD, Apo) and ×100 Plan Apo 1.4 NA objective. All image acquisition was managed through NIS-Elements software (Nikon Instruments).

Electron microscopy. For experiments conducted at OHSU, mice were killed and temporal bones were dissected; the inner-ear tissue was immersed in 4% formaldehyde for 45 min. Following three washes in 1 x PBS the organ of Corti was dissected from the inner ear and further fixed in 4% formaldehyde for 15 min. Tissue was washed three times in 1 x PBS and permeabilized in 0.2% Triton X-100, 1.0% normal donkey serum and 0.1% BSA for 15 min. Tissue was washed and incubated in primary antibody (0.4 μg ml⁻¹, diluted in block) overnight at 4°C. Tissue was then incubated for 3 h in block containing goat anti-rabbit Alexa Fluor 488 (Invitrogen, catalog #A21206: 4 μg ml⁻¹) and CF633 phallidin (Biotium, catalog #00446: 1250). Lastly, tissue was washed in 1 x PBS and mounted in Everbrite (Electron Microscopy Sciences). Samples were imaged on the Zeiss LSM 710 confocal microscope (Carl Zeiss Microimaging, Jena, Germany). Tissue was dissected from the cochlea with the tip of a fine forceps, and the inner ear was gently flushed with ~0.3 ml of 2.5% glutaraldehyde fixative solution for 2 h at room temperature. The membrabanous labyrinth containing the cochlea and vestibular end organs was removed by dissection. Specimens were processed using three 1 h incubations with 100% acetone. After dehydration, specimens were then dehydrated with an ascending ethanol series and critical point dried using liquid CO2 as the transitional fluid. Specimens were sputter coated with carbon and platinum and viewed using a Hitachi S-4800 field-emission scanning electron microscope operated at 5 kV. For experiments conducted on OHSU, mouse temporal bones were removed and submerged in 4% paraformaldehyde. The inner ear was dissected from the temporal bone and forceps were used to make a small hole at the apex of the cochlea so fixative could fully penetrate the tissue. Additional dissection was performed to expose the sensory epithelium and the tectorial membrane was manually removed using fine forceps. The tissue was transferred to a solution containing 2.5% glutaraldehyde and 0.15 M cacodylate buffer at pH 7.4. Tissue was further processed using a osmium-thiocarbohydrazide19 method (OTOTO), which alternated three osmium (OsO4) incubations, each for 1 h, with 1% thiocarbohydrazide incubations for 20 min. Tissue was dehydrated with an ethanol series and critical point dried using liquid CO2. Samples were mounted on stubs and imaged using a FEI Sirion XL30 FE field-emission scanning electron microscope operated at 5 kV.

Scanning electron microscopy. For experiments conducted at the NIDCD, after removal of the inner ear from experimental mice, a small hole was made at the top of the cochlea with the tip of a fine forceps, and the inner ear was gently flushed with ~0.3 ml of 2.5% glutaraldehyde fixative solution for 2 h at room temperature. The membranous labyrinth containing the cochlea and vestibular end organs was removed by dissection. Specimens were processed using three 1 h incubations with 100% acetone. After dehydration, specimens were then dehydrated with an ascending ethanol series and critical point dried using liquid CO2 as the transitional fluid. Specimens were sputter coated with carbon and platinum and viewed using a Hitachi S-4800 field-emission scanning electron microscope operated at 5 kV.
Culture and biostic transfection of rat inner-ear tissue. Organ of Corti and vestibular tissues were dissected from postnatal days 0–2 rats and attached to collagen-coated flasks. Cultures were maintained for 1 day in DMEM medium containing 10% fetal bovine serum and 1% antibiotic-antimycotic. After 1 day, half of the media was replaced by fresh media supplemented with 5% fetal bovine serum and ampicillin (1.5 µg/ml - Sigma) and maintained at 37 °C and 5% CO2. For transfections, 50 µg of DNA were precipitated onto 25 µg of 1-µm gold particles and loaded into the Helios Gene Gun cartridges (BioRad). Tissue explants were transfected with the gene gun set at 90 psi of helium and maintained in culture for 18–48 h. Samples were fixed and counterstained for confocal microscopy as described above.

In utero electroporation of mouse inner-ear tissue. C57BL/6 males were crossed with CD1 females to generate embryos for transuterine microinjection. For in utero injection and electroporation, pregnant females with E11.5 embryos were anaesthetized with 2% isoflurane in air. Following the injection, a square wave electroporator (Protech International CY1215C) was used to deliver a pulse of 60–100 mA to the injected embryo. Once the transuterine and electroporation steps were complete, the abdominal wall was closed using an absorbable suture. Females were monitored 60 min postoperatively and once per 24 h until birth. Immunocytochemistry was carried out as described above.

Auditory brainstem response threshold. ABR thresholds for Myo3a, Myo3b and Espn-1 mutant mice were measured at the NIDCD52. Avertin (0.015 ml g−1 body weight) was injected intraperitoneally for anaesthesia before recording. A sound-attenuated chamber using an auditory-evoked potential diagnostic system, calibrated by the manufacturer (Intelligent Hearing Systems, Miami, FL, USA), was used for ABR recordings. Average responses were recorded using three subdermal needle electrodes placed at the forehead and mastoid locations. Alternating polarity click and tone-burst stimuli of 47 mC0 s and 5 ms duration, respectively, were used as click and tone-burst stimuli of 47 mC0 s and 5 ms duration, respectively. ABR thresholds were considered to be 95 dB SPL for subsequent analyses.

Targeted mass spectrometry of purified hair bundles. Hair bundles were purified from either P4 to P6 or P21 to P25 mouse utricles as described13. For mass spectrometry, four independent preparations of hair bundles were used for each of the two ages. In addition, four preparations of 10 utricles were obtained for each age. Trypsin-digested peptides were prepared by short SDS-polyacrylamide gel electrophoresis runs and in-gel digests and were analysed using a Thermo Electron Orbitrap Velos ETD mass spectrometer25. For 10 utricles, 5–6 ear-equivalents were digested for each sample. The digest was reconstituted in 50 mM dithiothreitol was added to a final volume of ~50 µl; the samples were heated to 65 °C for 15 min, followed by 5 min at 95 °C. By running proteins ~1 cm into a NuPAGE 4–12% Bis-Tris gel, proteins were separated from contaminants after washing with high-performance liquid chromatography (HPLC)-grade water (vortexed 30 s), 200 µl of 50 mM NH4HCO3/50 mM MeOH (vortexed 1 min), 200 µl of 50% 50% NH4HCO3/50% acetonitrile (vortexed 5 min) and 200 µl of 100% acetonitrile (vortexed 30 s). The remaining solution was removed and the gel pieces were dried briefly. Gel pieces were rehydrated with 100 µl of freshly made 25 mM DTT in 50 mM NH4HCO3, then were incubated for 20 min at 56 °C. After digesting the supernatant, 100 µl of 55 mM iodoacetamide in 50 mM NH4HCO3 was added for 20 min at room temperature in the dark. The supernatant was discarded and the gel pieces were washed twice with 400 µl HPLC-grade water. The washes were discarded and 200 µl of 50% 50% NH4HCO3/50% acetonitrile was added (vortexed 5 min). This wash was discarded and 200 µl 100% acetonitrile was added (vortexed 1 min). The supernatant was discarded and samples were dried in a vacuum concentrator. A stock solution of 50 mM NH4HCO3 to the stock aliquot with swirling to mix; this ProteaseMAX solution was kept on ice. A 1.5 ml aliquot of 0.01% ProteaseMAX/4 µl trypsin was made by adding 15 µl 1% ProteaseMAX (in 50 mM NH4HCO3) to 1,440 µl of 50 mM NH4HCO3, the solution was mixed, then 45 µl of a 200 ng µl−1 trypsin (Promega, Madison, WI, USA) solution was added. Trypsin digestion was allowed to proceed for 3 h at 37 °C. The digest solution was transferred to new tubes (25–40 µl of liquid from each tube); 30 µl of 2.5% trifluoroacetic acid (in HPLC-grade water) was added to gel pieces (vortexed 15 min). The solution was removed and combined with the initial digest solution. The solution was vortexed and then the combined solution was centrifuged for 10 min at 14,000 r.p.m. in a microcentrifuge. The supernatant was transferred to 0.45 µm filter tubes (Millipore Ultrafree centrifugal filters, #UFOCVH); samples were spun 5 min at 4,000 r.p.m. All samples were dried in the SpeedVac until virtually all solution was evaporated (~ 2 h), then were stored at –80 °C before mass spectrometry. MaxQuant version 1.4.1.2 was used with the Andromeda search engine to analyse the data53.54; relative iBAQ values were calculated for each protein by dividing the iBAQ for a protein by the sum of all iBAQ values, excluding contaminants, for a single combined run of six gel pieces55. The data are available via ProteomeXchange (http://www.proteomexchange.org) with identifier PXD002167, and technical aspects of the experiments were discussed and validated elsewhere56.
Peptide samples were analysed with an Orbitrap Fusion Tribrid mass spectrometer (Thermo Scientific) coupled to a Thermo/Dionex Ultimate 3000 Rapid Separation UPLC system and EasySpray nanosource. Samples were loaded onto an Acclaim PepMap C18, 5 μm particle, 100 μm x 2 cm trap using a 5 μl/min flow rate and then separated on an EasySpray PepMap RSLC C18, 2 μm particle, 75 μm x 25 cm column at a 300 nA/min flow rate. Solvent A was water and solvent B was acetonitrile, each containing 0.1% (v/v) formic acid. After loading at 2 B for 5 min, peptides were separated using a 55 min gradient from 7.5 to 30% B, 10 min gradient from 30 to 90% B and 6 min gradient at 90% B, followed by a 19 min re-equilibration at 2% B. Peptides were analysed using the targeted MS2 mode of the Xcalibur software in which the doubly or triply charged fragments of the four calibration samples was performed and tested for linearity around the measurement range. Peptides that did not perform linearly (R2 > 0.98) were excluded from analysis.

**COST7 cell filopodia analysis.** COST7 cells (ATCC CRL-1651; http://www.atcc.org/Products/All/CRL-1651) were trypsinized, plated on coverslips and maintained at 37 °C in DMEM supplemented with 10% fetal bovine serum. Cells were transfected using Lipofectamine transfection reagent (Invitrogen) according to manufacturer’s instructions and incubated for 24 h. Samples were then fixed for 20 min in 4% formaldehyde in PBS, permeabilized for 30 min in 0.5% Triton X-100 in PBS and counterstained or processed for immunofluorescence as described earlier. Relative pixel intensity of fluorescently tagged proteins along filopodia was determined using ImageJ (NIH) software. All measurements were performed using ImageJ. The mean grey value within a specified region of interest was used to represent the fluorescence intensity of mEmerald-ESPNL in each transfected cell.

**Homology analysis of the ARD domain of ESPN-1 and ESPNL.** Protein sequence alignment of the N-terminal ARD of mouse ESPN-1 (amino acids 1–334) and ESPNL (amino acids 1–334) were aligned using ClustalW2 (ref. 58). The estimated sequence homology for the ARD of both proteins was 54%, ESPN-1 and ESPNL protein secondary structure was predicted using PSIPRED59. Structural models of the ARD of mouse ESPN-1 and ESPNL proteins were generated using I-TASSER60 and the human tyrosin R protein as a template (PDB ID: 1n11). The models were aligned using the MatchMaker tool included in the Chimera software61. The root-mean-square deviation between the ESPN-1 and ESPNL structural model was of 1.76 Å for 330 residues.

**GST pull-down assays.** Recombinant GST–ESPNL ARD protein was expressed in Rosetta cells and purified from bacterial lysates by using glutathione-agarose beads (Thermo Scientific). GFP-tagged MYO3A and MYO3B pre-THDI, THDI and post-THDI domain fusion proteins were expressed, respectively, in COS7 cells and lysates were obtained by incubation of COS7 cells following we required with fusion constructs, lysates were prepared by brief sonication in ice-cold lysis buffer (CLB) (5 mM DTT, 50 mM Tris pH 7.4, 150 mM NaCl, 2 mM EDTA, 1% Triton X 100, 1 mM PMSF, 1 mM aprotinin and 1 mM leupeptin) and ultracentrifugation at 150,000g for 20 min. To test for interactions with Xmas and MYO3B tail domains, the same amount of GST–ESPNL ARD or GST alone was bound to glutathione-agarose beads for 1 h at 4 °C, followed by incubation with COS7 cell lysate expressing GFP-tagged Xmas and MYO3B tail domain fusion proteins, respectively, in CLB for 2 h. The glutathione-agarose beads were then washed three times with 1 × PBS. The final pellets were then resuspended in SDS-polyacrylamide gel electrophoresis buffer.

Co-precipitates obtained from the final step of GST pull-down or COS7 lysate supernatants were separated on NuPAGE Bis-Tris 4–12% gels (Invitrogen) and transferred to nitrocellulose membrane for analysis by protein immunoblotting. Rab7 polyubiquitin-anti-GFP antibodies (Invitrogen) were used to detect GFP-tagged MYO3A and MYO3B tail domain fusion proteins in the lysate and pull-down fractions; rabbit polyclonal anti-GST (Calbiochem) antibodies were used to detect GST–ESPNL ARD or GST alone in purified and GST pull-down fractions. All immunoblots were visualized using horseradish peroxidise-linked goat anti-rabbit secondary antibodies (Cell Signaling) and LumiGLO chemiluminescent substrate (Cell Signaling).

**Experiment repeats.** Figure 1b–e: Confocal of Espn-1/1’’ bundle. Experiment repeated at least six times. Figure 1f–j: SEM of Espn-1/1’’ bundle. Experiment repeated two times, each three animals of each genotype. Figure 1k & l TEM of Espn-1/1’’ bundle. Experiment repeated two times, with three of each genotype. Figure 1n–p: Immunofluorescence of MYO3B in WT; immunofluorescence of MYO3B in Espn-1/1’’, IF of PB538 on Espn-1/1’’ bundle. Experiment repeated at least three each.

**Figure 3a–f: Immunofluorescence of Espn-1 and EspnL and Espn-1/1’’ bundle repeated more than five times. Figure 3g–h: Super-resolution. Experiment repeated two times. Figure 3i: Espn-1/1’’, ESPN-L. Experiment repeated four times. Figure 4b–e: Espn-1 and EspnL COS7 transfections. Experiment repeated more than five times. Figure 4g: Antibody testing. Experiment repeated once by immunostaining and once by protein immunoblot. Figure 4j–k: SEM of Espn-1 knockout. Experiment repeated two times.

**Figure 5d:e: ESPN-ARD + MYO3A and ESPN-L-ARD + MYO3B. Experiment repeated more than three times.**

**Figure 6a:d: Immunofluorescence of MYO3A and MYO3B. Experiment repeated more than six times.** Figure 6b:c: Confocal and SEM of MYO3A. Experiment repeated two times. Figure 6d, h, j: PCR with reverse transcription (RT–PCR). Experiment repeated two times. Figure 6d, k: ABR. Repeat information in legend. Figure 6f–h: confocal of bundle morphology in Mys3a and Mys3b null mice. Experiment repeated more than six times, with total numbers of bundles examined listed in the legend.

**Figure 7: COST7 transfections were all repeated more than six times.**

**References.**

1. Mattila, P. K. & Lappalainen, P. Filopodia: molecular architecture and cellular functions. *Nat. Rev. Mol. Cell Biol.* 9, 446–454 (2008).

2. Nambiar, R., McConnell, R. E. & Tyska, M. J. Myosin motor function: the ins and outs of actin-based cell motility. *Cell. Mol. Life Sci.* 67, 1239–1254 (2010).

3. Tilney, L. G., Tilney, M. S. & DelRosier, D. J. Actin filaments, stereocilia, and hair cells: how cells count and measure. *Annu. Rev. Cell Biol.* 8, 257–274 (1992).

4. Manor, U. & Kachar, B. Dynamic length regulation of sensory stereocilia. *Semin. Cell Dev. Biol.* 19, 502–510 (2008).

5. Jacobo, A. & Hudspeth, A. J. Reaction-diffusion model of hair-bundle morphogenesis. *Proc. Natl Acad. Sci. USA* 111, 15444–15449 (2014).

6. Orly, G., Naoz, M. & Gov, N. S. Physical model for the geometry of actin-based hair bundles. *Phys. Rev. Lett.* 101, 058106 (2008).

7. Orly, G., Manor, U. & Gov, N. S. A biophysical model for the staircase geometry of stereocilia. *PLoS ONE* 10, e0127926 (2015).

8. Dorer, A. A. & Avraham, K. B. Hearing loss: mechanisms revealed by genetics and cell biology. *Annul. Rev. Genet.* 43, 411–437 (2009).

9. Rzadzinska, A., Schneider, M., Noben-Trauth, K., Bartles, J. R. & Kachar, B. Balanced levels of Espn-1 are critical for stereociliary growth and length maintenance. *Cell Motil. Cytoskeleton* 62, 157–165 (2005).

10. Shin, J. B. et al. Molecular architecture of the chick vestibular hair bundle. *Nat. Neurosci.* 16, 365–374 (2013).

11. Belyantseva, I. A. et al. Myosin-XVa is required for tip localization of whorl and differential elongation of hair-cell stereocilia. *Nat. Cell Biol.* 7, 148–156 (2005).

12. Manor, U. et al. Regulation of stereocilia length by myosin XVa and whorl depends on the actin-regulatory protein whorl. *PLoS ONE* 8, e78863 (2013).
13. Salles, F. T. et al. Myosin IIIa boosts elongation of stereocilia by transporting espin 1 to the plus ends of actin filaments. Nat. Cell Biol. 11, 443–450 (2009).

14. Merritt, R. C. et al. Myosin IIIIB uses an actin-binding motif in its espin-1 cargo to reach the tips of actin protrusions. Curr. Biol. 22, 320–325 (2012).

15. Schneider, M. É. et al. A new compartment at stereocilia tips defined by spatial and temporal patterns of myosin III expression. J. Neurosci. 26, 10243–10252 (2006).

16. Loomis, P. A. et al. Espin cross-links cause the elongation of microvillus-type parallel actin bundles in vivo. J. Cell Biol. 163, 1045–1055 (2003).

17. Sekerkova, G. et al. Novel espin actin-bundling proteins are localized to Purkinje cell dendritic spines and bind the Src homology 3 adapter protein insulin receptor substrate p35. J. Neurosci. 23, 1310–1319 (2003).

18. Zheng, L., Beeler, D. M. & Barlow, R. J. Characterization and regulation of an additional actin-filament-binding site in large isoforms of the stereocilia actin-bundling protein espin. J. Cell Sci. 127, 1306–1317 (2014).

19. Walsh, T. et al. From flies’ eyes to our ears: mutations in a human class III myosin cause progressive nonsyndromic hearing loss DFNB30. Proc. Natl Acad. Sci. USA 99, 7518–7523 (2002).

20. Naz, S. et al. Mutations of ESPN cause autosomal recessive deafness and vestibular dysfunction. J. Med. Genet. 41, 591–595 (2004).

21. Zheng, L. et al. The deaf jerker mouse has a mutation in the gene encoding the espin actin-bundling proteins of hair cell stereocilia and lacks espins. Cell 102, 377–385 (2000).

22. Li, A., Xue, J. & Peterson, E. H. Architecture of the mouse utricle: the macula of hearing and peripheral innervation patterns in the utricular macula. J. Cell Sci. 127, 1306–1317 (2014).

23. Goldberg, J. M., Desmadryl, G., Baird, R. A. & Fernandez, C. The vestibular nerve of the chick. V. Relation between afferent discharge properties and peripheral innervation patterns in the utricular macula. J. Neurophysiol. 63, 791–804 (1990).

24. Gillespie, P. G. & Hudspeth, A. J. High-purity isolation of bullfrog hair bundles and subcellular and topological localization of constituent proteins. J. Cell Biol. 114, 625–640 (1991).

25. Krey, I. F., Sherman, N. E., Jeffery, E. D., Choi, D. & Barr-Gillespie, P. G. The proteome of mouse vestibular hair bundles over development. Sci. Data 2, 150047 (2015).

26. Lesur, A. & Domon, B. Advances in high-resolution accurate mass spectrometry application to targeted proteomics. Proteomics 15, 880–890 (2015).

27. Glenny, I. R. J., Kaulius, P., Matsudaia, P. & Weber, K. F-actin binding and bundling properties of fimbrin, a major cytoskeletal protein of microvillus core filaments. J. Biol. Chem. 256, 9283–9288 (1981).

28. Pyzocha, N. K., Ran, F. A., Hsu, P. D. & Zhang, F. RNA-guided genome editing of the kinase domain activation loop. J. Biol. Chem. 285, 35770–35782 (2010).

29. Quinten, O. A. et al. Myosin-3A kinase activity is regulated by phosphorylation of the kinase domain activation loop. J. Biol. Chem. 288, 37126–37137 (2013).

30. Dose, A. C. et al. Myo3A, one of two class III myosin genes expressed in vertebrate retina, is localized to the calycal processes of rod and cone photoreceptors and is expressed in the sacculus. Mol. Biol. Cell 14, 1058–1073 (2003).

31. Manor, U., Grati, M., Yengo, C. M., Kachar, B. & Gov, N. S. Competition and regulation of stereocilia lengths. J. Cell Sci. 127, 1306–1317 (2014).

32. A.M.W. examined the ears of f0 pups from a Tg(Espn-LacZ) strain obtained from OHSU Transgenic Mouse Model Shared Resource; and confocal microscopy from Laboratory of Neurobiology at the University of Virginia W.M. Keck Biomedical Mass Spectrometry Lab and the OHSU Proteomics Shared Resource (partial support from NIH core grants P30 EY010572 and P30 CA065353; Orbitrap Fusion Flexor or Orbitrap Fusion Lumos, with MORN repeat-containing adaptor proteins. Nat. Commun. 11, 297–305 (2015).

33. Manor, U., Grati, M., Yengo, C. M., Kachar, B. & Gov, N. S. Competition and regulation of stereocilia lengths. J. Cell Sci. 127, 1306–1317 (2014).

34. Dose, A. C. et al. A quantitative survey of gravity receptor function in mutant inner ears. J. Neurosci. 29, 112, 791–804 (1990).

35. Mecklenburg, K. L. et al. Intermolecular autophosphorylation regulates myosin IIIA motor. J. Cell Biol. 201, 91–97 (1981).

36. Dumont, R. A., Zhao, Y. D., Holt, J. R., Bahler, M. & Gillespie, P. G. Myosin-1 isoforms in neonatal rod and cone retinal photoreceptors and is expressed in the sacculus. J. Biol. Chem. 285, 375–389 (2002).

37. Krey, J. F. et al. Accurate label-free protein quantitation with high- and low-resolution mass spectrometers. J. Proteome Res. 13, 1034–1044 (2014).

38. Larkin, M. A. et al. Clustal W and Clustal X version 2.0. Bioinformatics 23, 2947–2948 (2007).

39. Jones, D. T. Protein secondary structure prediction based on position-specific scoring matrices. J. Mol. Biol. 292, 195–202 (1999).

40. Roy, A., Kucukural, A. & Zhang, Y. 1-TASSER: a unified platform for automated protein structure and function prediction. Nat. Protoc. 5, 725–738 (2010).

41. Meng, E. C., Pettersen, E. F., Couch, G. S., Huang, C. C. & Ferrin, T. E. Tools for integrated sequence-structure analysis with UCSF Chimera. BMC Bioinformatics 7, 339 (2006).

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Author contributions

S.E. and M.R.A. designed and performed experiments, acquired and analysed the data, and wrote the manuscript. M.G. helped design and make the Myo3A and Espin-1 knockouts. J.E.K. designed, carried out, and analysed the mass spectrometry experiments. A.M.W. examined the Myo3A knockouts. A.D.S. helped design the Espin-1 knockout. A.M.W. carried out sequence comparison of Espin-1 and EspnL. R.D. bioinformatic methods. R.C. performed experiments. B.A.M. helped with image analyses. F.T.S. helped make the Espin-1 knockout. M.A.B. and M.W.D. made tagged ESPNL constructs. S.M.J. performed vesicle functional analyses. D.C. carried out statistical analyses. I.D. helped make the Myo3A and Espin-1 knockouts. M.R. and C.M.Y. helped carry out co-immunoprecipitation.
assays. P.G.B.-G. and B.K. supervised the project, designed and performed experiments, acquired and analysed the data, and wrote the manuscript.

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Corrigendum: Stereocilia-staircase spacing is influenced by myosin III motors and their cargos espin-1 and espin-like

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