Whirlin interacts with espin and modulates its actin-regulatory function: an insight into the mechanism of Usher syndrome type II

Le Wang1,2, Junhuang Zou1, Zuolian Shen1, E. Song2 and Jun Yang1,3,*

1Department of Ophthalmology and Visual Sciences, Moran Eye Center, University of Utah, Salt Lake City, UT 84132, USA 2The first affiliated hospital of Jilin University, Changchun, Jilin, China and 3Department of Neurobiology and Anatomy, University of Utah, Salt Lake City, UT 84132, USA

Received July 30, 2011; Revised September 29, 2011; Accepted October 28, 2011

Whirlin mutations cause retinal degeneration and hearing loss in Usher syndrome type II (USH2) and non-syndromic deafness, DFNB31. Its protein recruits other USH2 causative proteins to form a complex at the periciliary membrane complex in photoreceptors and the ankle link of the stereocilia in hair cells. However, the biological function of this USH2 protein complex is largely unknown. Using a yeast two-hybrid screen, we identified espin, an actin-binding/bundling protein involved in human deafness when defective, as a whirlin-interacting protein. The interaction between these two proteins was confirmed by their coimmunoprecipitation and colocalization in cultured cells. This interaction involves multiple domains of both proteins and only occurs when espin does not bind to actin. Espin was partially colocalized with whirlin in the retina and the inner ear. In whirlin knockout mice, espin expression changed significantly in these two tissues. Further studies found that whirlin increased the mobility of espin and actin at the actin bundles cross-linked by espin and, eventually, affected the dimension of these actin bundles. In whirlin knockout mice, the stereocilia were thickened in inner hair cells. We conclude that the interaction between whirlin and espin and the balance between their expressions are required to maintain the actin bundle network in photoreceptors and hair cells. Disruption of this actin bundle network contributes to the pathogenic mechanism of hearing loss and retinal degeneration caused by whirlin and espin mutations. Espin is a component of the USH2 protein complex and could be a candidate gene for Usher syndrome.

INTRODUCTION

Usher syndrome is the most common genetic cause for the combined vision and hearing loss (1–3). Among its three clinical types, type II (USH2) is predominant and accounts for ~70% of all Usher cases. It is manifested as retinitis pigmentosa and congenital moderate hearing loss. Currently, usherin (4), G protein-coupled receptor 98 (GPR98) (5) and whirlin (6) have been identified as the USH2 causative genes. The proteins of these genes are known to bind to each other in vitro through PDZ (postsynaptic density 95; discs large; Zonula occludens-1) domain-mediated interactions (7–9). They colocalize at the periciliary membrane complex (PMC) in photoreceptors and the stereociliary ankle-link in hair cells (8–11). Mutations in one of the three USH2 genes lead to mislocalization of the other two proteins in mice (9,11), while delivery of whirlin back into whirlin knockout photoreceptors can rescue the localization of usherin and GPR98 (12). Therefore, the three USH2 proteins form a complex in vivo and defects in this complex are the primary cause for USH2 pathogenesis. However, the biological function of this complex is little known.

Among the three USH2 proteins, whirlin has PDZ domains and a proline-rich (PR) region (Fig. 1A), which are both protein–protein interaction domains. It is believed that whirlin is a scaffold protein and implicated in the assembly of the USH2 protein complex. At present, whirlin has been reported to interact with several proteins other than usherin.*

*To whom correspondence should be addressed at: John A Moran Eye Center, University of Utah, 65 Mario Capecchi Drive, Bldg 523, Salt Lake City, UT 84132, USA. Tel: +1 8012132591; Fax: +1 8015878314; Email: jun.yang@hsc.utah.edu

© The Author 2011. Published by Oxford University Press. All rights reserved. For Permissions, please email: journals.permissions@oup.com
and GPR98. In hair cells, whirlin associates with myosin XVa, Eps8 and p55 (13–17). In the shaker 2 mouse, which has a mutation in myosin XVa, whirlin, Eps8 and p55 are all mislocalized. In the whirler mouse, which has a mutation in whirlin, the expression of p55 and Eps8 is ablated or reduced, but myosin XVa expression is unchanged. These findings illustrate that myosin XVa is essential for the delivery of whirlin, Eps8 and p55, and whirlin is probably an adaptor between myosin XVa and its cargos. However, myosin XVa, Eps8 and p55 are all present at the tip but not the ankle-link of the stereocilia. Therefore, they are unlikely to be components of the USH2 complex. In photoreceptors, SANS (18), Cav1.3 alpha (19) and lia. Therefore, they are unlikely to be components of the USH2 complex in order to understand the USH2 pathogenesis.

**RESULTS**

**Whirlin interacts with espin**

A yeast two-hybrid screen of a mouse retinal cDNA library was conducted using a whirlin fragment containing the N-terminal two PDZ domains as the bait (Fig. 1A). An in-frame espin C-terminal fragment starting from its second PR, similar to espin 3, was identified to interact with whirlin (Fig. 1B). To further confirm the interaction between whirlin and espin, we did coimmunoprecipitation using recombinant whirlin and GFP-fused espin in HEK293 cells (Fig. 2A), which has no endogenous whirlin or espin expression (data not shown). In cells double transfected with whirlin and GFP-espin, the whirlin antibody precipitated GFP-espin together with whirlin, while the control non-immune rabbit immunoglobulin did not (Fig. 2A). As an additional negative control, the whirlin antibody did not precipitate GFP-espin in cells transfected only with GFP-espin (Fig. 2A).

In whirlin-transfected cells, whirlin with mCherry, GFP or no tag exhibited a diffuse signal pattern (Fig. 2B), and whirlin tagged with DsRed exhibited various aggregates (data not shown), probably due to the tendency of DsRed tetramerization. In espin-transfected cells, espin or espin tagged with GFP displayed various fibrous structures (Fig. 2B). When whirlin and espin were co-transfected, their signal patterns changed slightly. This change was the most obvious in cells co-transfected with GFP-espin and DsRed-whirlin, because the aggregation feature of DsRed-whirlin signals facilitated the observation. In these co-transfected cells, GFP-espin showed DsRed-whirlin-like aggregates as well as its original fibers, and DsRed-whirlin formed espin-like fibers as well as its original aggregates (Fig. 2C). Because the aggregation of DsRed-whirlin may cause artifacts, cells co-transfected with espin and whirlin with other tags or no tag were also examined. In these cells, whirlin signals sometimes displayed weak fibers, which were colocalized with espin fibers, and filament-binding sites at its N- and C-terminal ends. It functions in cross-linking actin filaments in an actin bundle in a calcium-insensitive way (29). Espin is shown to elongate filopodia, together with myosin IIIa, in fibroblast cells (28), microvilli in differentiated epithelial cells (27) and stereocilia in hair cells (28). All these cellular organelles are filled with parallel actin bundles. In hair cells, espin is localized along the entire length of stereocilia (25,28). In espin mutant mouse (the jerker mouse), the stereocilia fail to grow in width in various types of hair cells (30). Therefore, espin plays a role in defining the geometry of actin bundles in cells.

Here, we discovered a direct interaction between whirlin and espin. Whirlin exhibited an inhibitory role against the actin-binding/bundling activity of espin. Considering the very recent discovery of the interaction between whirlin and another actin-regulatory protein, Eps8 (16), our finding provides an alternative pathway for whirlin to regulate actin filament bundles in vivo. More importantly, espin is the first protein, other than the three USH2 proteins, to be found to have abnormal expression in both whirlin knockout photoreceptors and hair cells. This suggests that the alteration in espin expression contributes to the pathogenesis of USH2.
Figure 2. Whirlin and espin interact and are partially colocalized in cells. (A) GFP-espin is present in the whirlin immunoprecipitate (IP) from HEK293 cells double-transfected with whirlin and GFP-espin, but not from cells single-transfected with GFP-espin. Additionally, a rabbit non-immune immunoglobulin (IgG) did not immunoprecipitate GFP-espin from cells double-transfected with whirlin and GFP-espin. In the bottom, the western blot of whirlin shows the success of the immunoprecipitation procedure. (B) Distribution of recombinant whirlin and espin in their respective single-transfected COS-7 cells. (C) Partial colocalization of recombinant whirlin and espin in their double-transfected COS-7 cells as shown by confocal laser scanning microscopy. The signals of the two proteins in the white boxes are zoomed and shown on the right of the merged whole cell images. The scatter plots and Pearson’s coefficients of the two protein signals in the white boxes are in the right column. (D) Localization of whirlin at the base of filopodia filled with espin (arrowheads). The images labeled with only the protein’s names are from cells transfected with no tagged proteins and detected by immunostaining. The images with the protein’s names and various tags are from cells transfected with tagged proteins and detected by the fluorescence of tags. Scale bars, 10 µm in (B) and (C); 5 µm in (D).
espin showed some diffuse signals (Fig. 2C). A careful examination of the two protein signals revealed their partial colocalization. The correlation of the signal intensities from the two proteins in the same pixel was very strong in the region of cytoplasm with no thick espin fibers. The Pearson’s coefficients in these regions are in the range between 0.7 and 0.9 (Fig. 2C, right) (31). Interestingly, filopodia are a cellular structure similar to the stereocilia in hair cells. We frequently saw whirlin colocalized with espin at the base of filopodia, while espin was distributed along the entire filopodia (Fig. 2D). This partial colocalization pattern between whirlin and espin at the filopodia resembled that in hair cell stereocilia (see below). In summary, the partial colocalization between whirlin and espin, shown here, suggests that whirlin and espin interact with each other and that not all of these two proteins participate in their interaction.

**Whirlin binds to actin-free espin**

Because espin is an actin-binding/bundling protein, we investigated whether whirlin, espin and actin formed a ternary complex. Whirlin has been demonstrated to be able to interact with several actin-binding proteins in hair cells (13–17) and appears to be related with actin filaments (32,33). Thus, we first examined whether whirlin interacted directly with actin monomers or filaments. In whirlin-transfected cells, whirlin did not colocalize with actin filaments stained with phalloidin (Fig. 3A). In addition, whirlin was not coimmunoprecipitated with actin from whirlin-transfected cells (data not shown) or the mouse retina (Fig. 3B). These results were consistent with the notion that whirlin has no actin-binding activity.

In espin-transfected cells, espin fibrous structures were in fact actin bundles as revealed by phalloidin staining, indicating that they are espin cross-linked actin bundles (Fig. 3C). In espin and whirlin double-transfected cells, the espin fibrous structures remained stained with phalloidin. However, whirlin showed very weak signals along these espin/actin bundles (Fig. 3D). This finding suggested that whirlin may not interact with actin when espin cross-linked actin filaments. Cytochalasin D is known to bind to the barbed, fast growing end of actin filaments and to inhibit actin polymerization (34). It has been widely used to disassemble actin filaments in cultured cells. We found that cytochalasin D treatment before immunoprecipitation, which is thought to release espin when the actin bundles were disrupted, increased 4-fold the amount of GFP-fused espin in the whirlin immunoprecipitate (compare Figs 2A and 3E). This result is consistent with our colocalization finding (Figs 2C and 3D) and supports the concept that the interaction between whirlin and espin occurs when espin does not bundle the actin filaments.

Besides cross-linking actin filaments, espin is able to bind to actin monomers through its WH2 domain. Then, we asked whether whirlin was able to associate with actin monomers through its interaction with espin. In espin-transfected cells, espin was able to coimmunoprecipitated with actin (Fig. 3F). In whirlin and espin double-transfected cells, the whirlin antibody was able to immunoprecipitate espin (Figs 2A and 3E) and the interaction between espin and actin (Fig. 3F). However, in the cytochalasin D-treated cells double transfected with whirlin and espin, we still could not coimmunoprecipitate whirlin and actin (Fig. 3G). Taken together, whirlin is not able to associate with actin either directly or indirectly through espin. It only interacts with actin-free espin.

**Multiple regions of whirlin and espin are involved in their interaction**

To learn more about the interaction between whirlin and espin, five espin fragments were tested for their abilities to interact with full-length whirlin (Fig. 4A). Compared with WH2L, WH2H has 25 extra amino acids that are unique in espin 4. In a yeast two-hybrid analysis, espin AR was able to bind to whirlin no matter it was in the prey or bait vector (Fig. 4B), while espin WH2L, WH2H or ABM was not. The interaction between whirlin and espin PR could not be determined, because espin PR in the prey and bait vector behaved abnormally (see next paragraph). However, because the espin fragment identified originally from our yeast two-hybrid screen contained a PR region but not the AR region (Fig. 1B), we believed that espin PR was involved in the interaction between whirlin and espin. Supportively, individual coimmunoprecipitations of espin AR and PR with whirlin were found in HEK293 cells (Fig. 4C). These two espin fragments also showed colocalization with whirlin in the transfected cells (Fig. 5). Surprisingly, to some degree, espin ABM also displayed colocalization with whirlin in their double-transfected cells (Fig. 5). This suggested that espin ABM was likely to directly or indirectly associate with whirlin as well. Because both espin WH2H and WH2L fragments did not colocalize with whirlin, the espin WH2 domain could probably inhibit the association between espin ABM and whirlin. In summary, the results from the yeast two-hybrid, coimmunoprecipitation and colocalization analyses demonstrate that multiple domains in espins 1–3 are involved in their interaction with whirlin and that espin 4 probably does not interact with whirlin.

Two whirlin fragments were examined for their interactions with espin, the whirlin N- and C-terminal halves (Fig. 6A). Similar to our initial yeast two-hybrid screen result, the whirlin N-terminal half was shown to strongly bind to espin AR (Fig. 6B). However, the interaction of whirlin fragments with espin PR could not be determined using this technique for the following reasons. Espin PR in the prey vector was able to grow on the selective medium with the empty bait vector, indicating that it was able to self-activate in the prey vector (#8 in Fig. 6B). Additionally, when espin PR was in the bait vector, it did not grow on the selective medium even with whirlin full-length in the prey vector (#13 in Fig. 6B), which we considered a positive control. Therefore, espin PR in the bait vector could be toxic to the yeast host. In cultured cells, we could not coimmunoprecipitate either whirlin fragment with espin (data not shown). The colocalization between whirlin fragments and espin was also not as strong as that between full-length whirlin and espin (Fig. 6C). The Pearson’s coefficients were below 0.56.
Therefore, we believe that the interaction between whirlin and espin requires full-length whirlin.

**Both whirlin and espin are expressed in multiple neural tissues**

Western blotting analysis demonstrated that whirlin was expressed in various brain tissues (Fig. 7A), including the cerebrum, cerebellum, brain stem as well as the retina and the inner ear (9). The bands in non-neural tissues were believed to be non-specific, because they had the same molecular weight in the wild-type mouse and the two whirlin mutant mouse lines, the whirlin knockout and the whirler mice. These two whirlin mutant mice are known to have different large deletions in the gene, causing premature truncations of the whirlin protein (9,32). By the same technique, espin was shown to be present in multiple tissues, including the cerebrum, cerebellum, brain stem, retina and many other non-neural tissues (Fig. 7B). It expressed different isoforms with various molecular sizes in different tissues. Consistent with previous findings, espin expressed a 110 kDa band in the testis and a 30 kDa band in the kidney in our western blot (29). Together with the known expression of espin in the inner ear (35), the results from this western
blotting analysis suggest that the interaction between whirlin and espin may exist in various brain tissues, the retina and the inner ear. Partial colocalization of whirlin and espin is observed in photoreceptors and hair cells. Because mutations in whirlin and espin cause hearing loss (23–25,32) and mutations in whirlin also lead to retinal degeneration (6,9) in both humans and mice, we focused our studies on their interaction in the retina and the inner ear. Espin previously was localized at the outer limiting membrane in the retina and thought to be present at the microvilli of the Muller cells (35). We did in situ hybridization using a pan-espin probe and found that, compared with the sense control, espin mRNA was abundant in both the inner nuclear layer and the ganglion cell layer (Fig. 8A), suggesting that espin is present in multiple cell types in the retina. In photoreceptors (the outer nuclear layer), we detected weak signals, indicating espin is expressed in photoreceptors as well. Immunostaining of the lightly fixed mouse retina detected espin protein signals in the inner segment and the synaptic end of photoreceptors (the inner segment layer and the outer plexiform layer, respectively) (Fig. 8B). At the high magnification, weak bar-shaped espin signals were seen at the top of the inner segment, marked by rootletin signals, and very close to the connecting cilium, marked by retinitis pigmentosa GTPase regulator (RPGR) (Fig. 8C). Double staining showed the colocalization of whirlin with the bar-shaped espin signal at the PMC (Fig. 8C). In hair cells, double staining localized espin along the entire stereocilia and whirlin at the tip and the ankle-link (the base) of the stereocilia (Fig. 8D). The two proteins were colocalized at the ankle-link but not the tip of the stereocilia, similar to the colocalization pattern of whirlin and espin at the filopodia in cultured cells (Fig. 2D). Taken together, the partial colocalization between whirlin and espin indicates that the interaction between the two proteins exists in photoreceptors and hair cells.

Espin expression is altered in the whirlin knockout retina and inner ear

To investigate the interaction between whirlin and espin in vivo, we examined espin expression in the whirlin knockout retina and hair cells. Espin is known to have three isoforms expressed in the retina, espins 1, 3 and 4 (35). In our hand, the espin antibody (espin_JB), published previously (35–37), detected espins 1 and 3 in the retina, and a commercial espin antibody (espin K-14) detected all three espins plus two non-specific bands (Fig. 9A). Using these espin antibodies, we consistently found that, in whirlin knockout retinas, the expression level of espin 3 decreased to ≏10%, while that of espin 4 increased by ≏10-fold. The expression of espin 1 did not change significantly. As we already showed above, whirlin was able to interact with espins 1–3. Therefore, the decrease in espin 3 expression could result from the absence of whirlin in the retina, and the increase in espin 4 expression could be a compensatory response to the decrease in espin 3. Immunostaining revealed no changes in the espin signal pattern and intensity in the whirlin knockout retina, compared with those in the wild-type retina, at either the low (Fig. 9B) or the high (Fig. 9C) magnification. This result could be attributed to the similar fold but opposite direction of changes in espin 3 and 4 expression and the absence of signal pattern changes in the whirlin knockout retina.

Because of the technical difficulty to run western blotting analysis using the inner ear tissue in our lab, we examined the expression of espin in hair cells by immunostaining (Fig. 9D). In the whirlin knockout outer hair cells, the intensity of espin signals significantly decreased to ≏30% of that in the...
Figure 5. Multiple espin fragments colocalize with whirlin in cells as shown by confocal laser scanning microscopy. Five GFP-tagged espin fragments were individually cotransfected with mCherry-tagged whirlin in COS-7 cells. GFP-tagged rootletin was used as a negative control (bottom row). Colocalization of GFP and mCherry fluorescent signals was analyzed in the region marked by white boxes. The signals from individual and merged channels in the white boxes are displayed on the right of the merged images of whole cells. The scatter plots and the Pearson’s coefficients of the GFP and mCherry signals in the white boxes are shown in the right column. Scale bars, 10 μm.
wild-type, while the area of espin signals at the stereocilia in each hair cell remained unchanged. Considering that espin is localized along the entire stereocilia in hair cells (Fig. 8D) and that the length of stereocilia does not decrease significantly in whirlin knockout mice (9), these results indicate that, in whirlin knockout mice, espin signals are still present along the entire stereocilia, but their expression level is tremendously decreased in the outer hair cells. The changes of espin expression in the inner hair cells appeared similar to those in the outer hair cells (Fig. 9D). Therefore, the alteration of espin expression in the retina and the hair cells in the absence of whirlin strongly suggests that the two proteins interact in vivo in these tissues.

Whirlin accelerates the espin mobility at the actin bundle in filopodia

To understand the functional significance of the interaction between whirlin and espin, we studied the effect of whirlin on espin mobility. We conducted fluorescence microscopy on live COS-7 cells expressing different whirlin and espin fragments.
recovery after photobleaching (FRAP) in COS-7 cells using the GFP-tagged espin. We focused on three espin-containing structures, the intracellular fibers, filopodia and star-like dots (Fig. 10A). At the filopodia, the espin signals displayed a different recovery dynamics from those at other structures. They showed a full and slow fluorescence recovery (Fig. 10B and C), indicating that all espin molecules are mobile in the actin bundle and that it takes long time to replace the bleached espin in the actin bundle with the free espin. The difference in the recovery dynamics of GFP-espin between the filopodia and other cellular structures could be attributed to the different composition and organization of espin cross-linked actin bundles in these structures. For example, some actin-binding/bundling proteins are specifically localized in the filopodia instead of the cytoplasm (38,39). Whirlin was found to significantly shorten the espin recovery time but had no effect on espin final recovery percentage at the filopodia (Fig. 10C). This suggests that whirlin accelerates the exchange between pools of free espin and espin bound with actin bundles at the filopodia and, thus, may indirectly weaken the cross-link
Figure 8. Whirlin and espin partially colocalize in photoreceptors and hair cells. (A) In situ hybridization demonstrates that, in the mouse retina, espin mRNA is abundant in the inner nuclear layer (INL) and the ganglion cell layer (GCL); it also exists in the outer nuclear layer (ONL), which is the photoreceptor layer. (B) Immunostaining localizes espin in the inner segment (IS) and the synaptic terminus (OPL) of mouse photoreceptors. (C) At the high magnification, confocal laser scanning microscopy displays that a fraction of espin signals are present next to retinitis pigmentosa GTPase regulator (RPGR) (arrows, top row), above rootletin (arrow, middle row) and colocalized with whirlin (arrows, bottom row). The localizations of RPGR, rootletin and whirlin in photoreceptors are shown in green in the cartoon on the top right. (D) In mouse hair cells at postnatal day 4, confocal laser scanning microscopy shows that espin is localized along the entire stereocilia, while whirlin is present at the ankle-link (the base) and the tip of the stereocilia. These two proteins are colocalized at the ankle-link of the stereocilia as indicated by the color yellow in the merged view. OS, outer segment; IPL, inner plexiform layer; CC, connecting cilium; PMC, periciliary membrane complex; BB, basal body; SC, stereocilia; AL, ankle-link; CB, cell body. Scale bars, 20 μm in (A) and (B), 5 μm in (C) and 2.5 μm in (D).
Figure 9. Espin expression is changed in the retina and the inner ear in whirlin knockout mice. (A) Western blotting analysis demonstrates that espin 3 expression decreased and espin 4 expression increased in the retina of whirlin knockout mice (Whrn^/^). Left, representative western blots. Espin K-14 AB is an espin antibody from Santa Cruz Biotechnology, Inc. No difference in actin expression was found in the retina between whirlin knockout and wild-type (WT) mice. Thus, actin signals were used as a loading control for the quantitative analysis of espin expression in the retina. ns, non-specific bands. Right, quantification of the espin band intensities. The numbers in the bottom of each bar are the numbers of animals analyzed. Error bars, the standard error of the mean; *P < 0.05; **P < 0.01. (B and C) Confocal laser scanning imaging did not reveal obvious changes in the espin signal distribution and intensity in the whirlin knockout retina compared with those in the wild-type retina at both the low (B) and the high (arrows, C) magnifications. (D) Left, representative espin immunostaining images of the wild-type and whirlin knockout hair cells. Right, bar charts comparing the espin signal intensities and areas in the inner and outer hair cells between the wild-type and whirlin knockout mice. Numbers in the bottom of each bar are numbers of hair cells analyzed from one to four animals. Error bars, the standard error of the mean. **P < 0.01. OS, outer segment; IS, inner segment; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer; OHC, outer hair cells; IHC, inner hair cells. Scale bars, 10 \( \mu \)m in (B) and (C), 5 \( \mu \)m in (D).
of espin with actin filaments. Whirlin was found not to significantly change the FRAP responses of espin at the intracellular fibers or star-like dots (Fig. 10B and C).

**Whirlin accelerates the actin mobility at the actin bundle cross-linked by espin**

To further investigate the whirlin effect on actin bundles cross-linked by espin, we performed FRAP in COS-7 cells using GFP-tagged actin (Fig. 11). When GFP-actin was single-transfected, signals from actin monomers and unbundled actin filaments were distributed uniformly in the cytoplasm (data not shown). The fluorescence recovery of these actin signals after the photobleaching was complete and very fast within seconds (Fig. 11B and C). When GFP-actin was double-transfected with espin, actin bundles were formed by espin cross-linking. Actin filaments in these bundles were dramatically stable. The photobleached actin signals at the extremely thick bundles (data not shown) or the strong star-like dots (Fig. 11A, bottom row), presumably the origins of actin polymerization, could not recover to a saturated phase within 2000 seconds. At the thin intracellular fibers (Fig. 11A, upper row), the final percentage and time of the GFP-actin recovery were decreased and prolonged, respectively (Fig. 11B and C). Moreover, the GFP-actin recovery time (Fig. 11B and C) was much longer than the GFP-espin recovery time (Fig. 10B and C) at the same cellular structure. This suggests that actin is much more static than espin in the espin cross-linked actin bundles, which could be explained by the fact that a single actin filament is cross-linked by several espin molecules in the bundle (40,41). Thus, espin stabilized actin filaments by cross-linking them into bundles at multiple sites.

When whirlin was cotransfected with both GFP-actin and espin, the recovery time was shortened by half and the final recovery percentage stayed the same at the intracellular thin fibers, compared with those in cells transfected only with GFP-actin and espin (Fig. 11B and C). Although we did not discover any effect of whirlin on the espin mobility at the intracellular espin/actin fibers using FRAP (Fig. 10C), based on the previous finding that whirlin does not bind to actin either directly or indirectly (Fig. 3), this result of FRAP using GFP-actin suggests that whirlin somehow indirectly influence the actin-bundling activity of espin and, thus, destabilize the actin bundles. We did not conduct GFP-actin FRAP on the actin bundles at the filopodia, because the treadmilling activity of the actin bundles and the extremely active movement of the filopodia did not allow us to monitor the entire process of fluorescence recovery.

**Whirlin shortens actin bundles cross-linked by espin in cultured cells**

In transfected COS-7 cells, recombinant espin showed the following main distinct signal patterns in the cytoplasm, the long fibrous network, the ~10 μm short fibers (including filopodia) and the star-like dots (Fig. 12A). These espin structures were also labeled by phalloidin (data not shown), indicating they are actin filament bundles cross-linked by espin. We compared the
percentage of cells with espin long fibers, short fibers or star-like dots in the absence and the presence of whirlin. We found that, in the presence of whirlin, cells with espin long fibers decreased, while cells with espin short fibers or star-like dots increased (Fig. 12B). Therefore, in contrast to espin, which elongates actin bundles (26), whirlin plays a role in reducing the length of espin-containing actin fibers. Espin is known not to elongate the filopodia in COS-7 cells (28). Interestingly, whirlin also had no effect on the length of filopodia filled with espin cross-linked actin bundles (data not shown). This indicates that whirlin does not reduce the actin bundle length directly but through its modulation of espin activity.

Ablation of whirlin causes thick stereocilia in the inner hair cells

To elucidate the potential consequence of changes in espin expression in whirlin knockout mice, we measured the diameter of the hair cell stereocilia and discovered that the whirlin knockout mice had thick stereocilia in the inner hair cell and normal stereocilia in the outer hair cell (Fig. 13). The difference in this phenotype between the inner and outer hair cells is not clear, but it could result from different biological functions, structures and molecular compositions in these two types of cells. Considering the findings of thin stereocilia in the jerker mouse (30) and thick stereocilia in the whirler mouse (33), we propose that both espin and whirlin are...
required to maintain the normal diameter of stereocilia in the wild-type mice and that they play opposite roles in this function.

DISCUSSION

This study has discovered an interaction between whirlin and espin and revealed its functional significance. Espin cross-links actin filaments and induces the formation of actin bundles. It stabilizes and elongates the actin bundles presumably through preventing these bundles from disassembly and actin filaments from depolymerization (26). Whirlin interacts with a pool of espin, which does not bind to actin monomers or filaments. This interaction activates the exchange rate of espin between its actin-free and actin-bound pools, destabilizes the espin cross-linked actin bundles and shortens these bundles. In whirlin mutant mice, the hair cell stereocilia show an opposite phenotype, compared with the espin mutant mice. All these findings suggest that the interaction between whirlin and espin weakens the actin cross-linking activity of espin. Whirlin and espin express in multiple neural tissues, suggesting their interaction may exist in these tissues. In photoreceptors and hair cells, whirlin and espin are colocalized at the PMC and the stereociliary ankle-link, and loss of whirlin leads to the changes in espin expression. These data strongly support the existence of the interaction between these two proteins in vivo. Whirlin is a scaffold protein in the USH2 protein complex. Therefore, the interaction between whirlin and espin indicates that espin is a component of the USH2 complex. The abnormal actin filament network caused by disruption of the interaction between whirlin and espin in hair cells and, perhaps, photoreceptors could be one of the reasons leading to the final hearing loss and retinal degeneration manifested in USH2 patients.

The stereocilia are rigid finger-like protrusions at the apex of hair cells, filled with tightly bundled and uniformly polarized actin filaments. Their organization and dimension are essential for normal sound detection (42–44), because the stereocilia are responsible for converting their deflection induced by the sound into electrical responses. Recent studies in deaf mutant mice demonstrate that actin-binding proteins and proteins of interstereociliary links function in the development and maintenance of normal stereocilia. Among these proteins, Eps8 (16,45), espin (26,30), myosin XVa, myosin IIIa, myosin VI, myosin VIIa (43), fimbrins/plastins (46,47), twinfilin 2 (48) and gelsolin (49) are actin-binding proteins. Whirlin, usherin, GPR98 (11), cadherin 23, protocadherin 15 (50–52) and protein tyrosine phosphatase receptor Q (PTPRQ) (53,54) are components of the interstereociliary links. Defects in these proteins result in abnormally short, long or fused stereocilia. It is now believed that myosin XVa transports Eps8 and whirlin, and myosin IIIa transports espin to the different subregions of the stereociliary tip (13–16,28), while myosin VI transports PTPRQ to the stereociliary base (54). Myosin VIIa is along the entire length of stereocilia. Its cargos have not been clearly demonstrated.

Unlike previous studies focusing on whirlin and espin at the stereociliary tip, we have found that they physically interact and are colocalized at the ankle-link at the base of the stereocilia. These results provide new insight into the regulatory mechanism for the stereociliary dimension, especially the stereociliary width. During development, the stereocilia first grow by forming a thin actin core inside them. Then, either sequentially or simultaneously, they increase their width and

Figure 13. Whirlin knockout mice have thick stereocilia in the inner hair cells. (A) Scanning electron microscopy showing the inner hair cells (arrows) in the wild-type (WT, top) and whirlin knockout (Whrn-/-, bottom) mice. The imaging conditions and the scale bars are displayed in the bottom of each image. (B) The diameter of inner hair cell (IHC) stereocilia increases in whirlin knockout mice. (C) The outer hair cell (OHC) stereocilia have a normal diameter in the whirlin knockout mice. The numbers in the bottom of each bar are the numbers of cells measured. Error bars, the standard error of the mean. ∗∗P < 0.01.
elongate to reach their final dimension in adult, by adding more actin filaments to the periphery and the distal end of the actin core, respectively (44). In the jerker mouse, stereocilia failed to grow in width, while the defect in the stereociliary length varies (30). Thus, the small diameter of stereocilium is the main defect caused by the espin mutation. In the whirler mouse, stereocilia were reported to be thick, while the actin filament packing density is normal (33). Here, we show that our whirlin knockout mouse also has thick stereocilia in the inner hair cell. Therefore, whirlin and espin play opposite roles in maintaining stereociliary diameter, with espin increasing and whirlin decreasing the stereociliary width. Supportively, espin was shown to increase the thickness of some microvilli in its transfected epithelium cells (55). In the whirlin knockout mouse, espin expression decreased significantly at the stereocilia. However, the thick inner hair cell stereocilia, observed in this mouse, indicates that the remaining residual espin is still able to increase the stereociliary diameter in the absence of whirlin, the inhibitory factor. Therefore, we believe that the balance between espin and whirlin expression is critical for the stereocilia to achieve their final normal diameter. Additionally, the stereociliary ankle-link, where whirlin is located, is a transient structure existing only during development (56), and the thinning of stereocilia in the jerker mouse starts at the time point when the ankle-link emerges (30). Therefore, the effect of espin and whirlin on the stereociliary width occurs primarily during development. Currently, the underlying mechanism to regulate the stereociliary width by espin and whirlin is not clearly understood. According to our data, the following model could be proposed. Using its actin-bundling activity, espin stabilizes the parallel actin bundle inside the stereocilia. At the same time, espin could recruit free actin filaments to the existing parallel actin bundles. By these means, espin could eventually widen the stereocilia. Whirlin at the ankle-link of the stereocilia interacts with the actin-free espin and accelerates the espin exchange between its actin-free and actin-bound pools. It also facilitates the dissociation of actin filaments from the parallel actin bundle. Therefore, whirlin could offset the activity of espin and reduce the stereociliary diameter.

Although the interaction between whirlin and espin was established here by a series of experiments, including yeast two-hybrid analysis, coimmunoprecipitation and colocalization, only a small amount of espin was found in the whirlin immunoprecipitate in the cultured cells (Figs 2A and 3E). This is probably due to the fact that whirlin only interacts with actin-free espin, as shown by communoprecipitation and immunostaining experiments (Fig. 3). Espin has an unusual high affinity for actin filaments, and it also binds to actin monomers (26). Therefore, there is only a small pool of actin-free espin in cells available for whirlin binding. Moreover, there is a possibility that whirlin and espin interact through a third protein. Up to now, no protein is known to interact with both whirlin and espin. Additionally, the chance that the same protein mediates the indirect association between whirlin and espin in all our biochemical assays is extremely low. Thus, we believe that the interaction between whirlin and espin found in this study is direct.

Multiple espin domains and full-length whirlin are involved in the interaction between whirlin and espin. Among the espin domains, AR consistently shows the interaction with whirlin. Espin AR can also bind to myosin IIIa, when myosin IIIa transports espin to the stereociliary tip (28). Therefore, the interaction between whirlin and espin at the base of the stereocilia may control the transport of espin. Besides binding to whirlin, espin PR can bind to IRSp53 and profilin (35). These interactions may also be affected by the interaction of espin with whirlin. Interestingly, one of the whirlin-interacting partners, Eps8 (16), can associate with IRSp53 as well (57). As a consequence, whirlin, espin, Eps8 and IRSp53 may form a complex under some specific condition in vivo. Furthermore, in jerker mice, myosin XVa localization is affected in hair cells (58). Myosin XVa is known to bind to whirlin and to transport it to the stereociliary tip. Therefore, the interaction between whirlin and espin may somehow affect the stereociliary tip complex of whirlin, Eps8 and myosin XVa. At this time, the regulatory mechanism for whirlin to interact only with the actin-free espin remains elusive. Posttranslational modifications of the binding sites in whirlin and espin, such as phosphorylation, could play a role. Additionally, the common binding site on espin shared between whirlin and actin and the mutual blockage of the interaction of espin with actin and whirlin are possible. For example, the ABM domain of espin could bind to both whirlin and actin filaments. Therefore, the binding of espin with any one of these two partners could block its binding to the other.

Espins 1 and 3 are the two major isoforms expressed in the inner ear around postnatal day 4 (36). At this developmental stage, one report shows that espin 1 is mainly in the vestibular system and espin 3 is the main isoform in the cochlear hair cell (35). Another report presents evidence that espin 1 is localized at the tip and other espins along the entire length of stereocilia in the cochlear hair cell (28). Despite the discrepancy between these two reports, they support the notion that espin 3 is the predominant isoform along the entire stereocilia. Therefore, the signals in our immunostaining of cochlear hair cells in vivo could be interpreted as a consequence, whirlin, espin, Eps8 and IRSp53 may form a complex under some specific condition in vivo. Furthermore, in jerker mice, myosin XVa localization is affected in hair cells (58). Myosin XVa is known to bind to whirlin and to transport it to the stereociliary tip. Therefore, the interaction between whirlin and espin may somehow affect the stereociliary tip complex of whirlin, Eps8 and myosin XVa. At this time, the regulatory mechanism for whirlin to interact only with the actin-free espin remains elusive. Posttranslational modifications of the binding sites in whirlin and espin, such as phosphorylation, could play a role. Additionally, the common binding site on espin shared between whirlin and actin and the mutual blockage of the interaction of espin with actin and whirlin are possible. For example, the ABM domain of espin could bind to both whirlin and actin filaments. Therefore, the binding of espin with any one of these two partners could block its binding to the other.

In photoreceptors, the PMC is localized on the apical plasma membrane of the inner segment, directly facing the connecting cilium (Fig. 8C, cartoon). Next to it, an actin bundle could originate from the basal bodies (27). It has been shown that the actin filament network in this region is essential for the integrity of the outer segment membrane disks (59). Additionally, the importance of this actin filament network is supported by the localization of myosin VIIa at the connecting cilium and the PMC (20,60–62), because myosin VIIa is an actin-based motor protein and is involved in Usher syndrome type I. Therefore, the interaction of espin and whirlin could be one of the crucial mechanisms to maintain the dynamics and organization of the actin filament network around the PMC. Disruption of this interaction might contribute to the final retinal degeneration in whirlin knockout mice and USH2 patients. Up to now, the alteration of espin expression is the first molecular phenotype, except the changes in USH2 proteins, found in the retina with mutations in USH2 genes. This study suggests that espin could be a candidate gene of human Usher syndrome.
MATERIALS AND METHODS

DNA plasmids, antibodies and animals

Whirlin full-length cDNA (3–907 amino acids, NP_082916) was cloned from the mouse retina into the pCDNA3.1(−), pEGFP-C2 and pDsRed2-C1 vectors. The whirlin tagged with mCherry plasmid was constructed by replacing the DsRed sequence with a mCherry sequence in the whirlin/pDsRed2-C1 plasmid. Whirlin N- and C-terminal fragments (3–472 amino acids and 438–907 amino acids, NP_082916) in the pGBK7T and pGADT7 vectors were described previously (9). In this study, the same whirlin N- and C-terminal fragments were cloned into the pEGFP-C2 vector. The AR (1–350 amino acids), PR (344–660 amino acids), WH2L (651–871 amino acids), WH2H (651–871 amino acids with 25 extra amino acids unique in espin 4) and ABM (710–871 amino acids) fragments of espin (NP_997570) were cloned from the mouse retina into the pEGFP-C2, pGBK7T and pGADT7 vectors. All DNA plasmids constructed in this study were confirmed by DNA sequencing. Rat espin plasmids with or without the GFP tag and the rootletin-GFP plasmid were published previously (41,63). Human beta-actin GFP in the pCDNA3 vector was obtained from Dr Beat Imhof, Universite de Geneve, Geneva, Switzerland.

The polyclonal rabbit espin, whirlin and rootletin antibodies, polyclonal guinea pig RPGR antibody and polyclonal chicken whirlin antibody were described previously (9,12,35–37,63). A second polyclonal rabbit espin antibody (K-14) was purchased from Santa Cruz Biotechnology, Inc (Santa Cruz, CA, USA). The rabbit and chicken antibodies against GFP were from Abcam (Cambridge, MA, USA). Hoescht dye 33342, Alexa fluorochrome-conjugated phalloidin, streptavidin and secondary antibodies were obtained from Invitrogen (Carlsbad, CA, USA). An aliquot of the rabbit polyclonal whirlin antibody was biotin-labeled according to the manufacturer’s instructions (FluoReporter® mini-biotin-XX protein labeling kit, Invitrogen). Whirlin knockout and whirler mice were described previously (9). All experiments involving animals were approved by the Institutional Animal Care and Use Committee at the University of Utah.

Yeast two-hybrid screen and analysis

Cloning vectors, yeast host cells and reagents were purchased from Clontech Laboratories, Inc. (Palo Alto, CA, USA). The yeast two-hybrid screen was performed using the Match maker™ Gold yeast two-hybrid system, a mouse retinal Mate & Plate™ library and a whirlin N-terminal fragment as the bait. The expression of the bait in the yeast host was confirmed by western blotting analysis using a Myc antibody. To screen whirlin-interacting proteins, the bait strain of yeast was mated with the library strain, according to the manufacturer’s manual. The mated culture was spread on both SD-2 (-Leu/-Trp) and SD-4 (-Leu/-Trp/-Ade/-His) plates. Calculation from the number of colonies grown on SD-2 plates indicated that ~10⁶ colonies were screened. The colonies grown on the SD-4 plates were selected and their prey DNA plasmids were rescued. The DNA inserts in the obtained prey plasmids were amplified by PCR and sequenced.

To examine the interactions between whirlin and espin fragments, cotransformation of these protein cDNA fragments in the bait (pGBK7T) and prey (pGADT7) vectors was performed. The cotransformed cultures were spread on SD-2 plates to assess the success of the cotransformation procedure and on SD-4 plates to analyze the existence of interactions. Growth on the SD-2 plates indicated that both bait and prey plasmids were successfully transformed into the same yeast host cell. Growth on the SD-4 plates indicated that the proteins carried in the bait and prey plasmids interacted.

Cell culture and cytochalasin D treatment

COS-7 and HEK293 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 5% fetal bovine serum (Invitrogen). Transient transfections were carried out using the TurboFect™ in vitro transfection reagent (Fermentas Life Sciences, Glen Burnie, MD, USA) according to the manufacturer’s instructions. Cells were collected at ~24 h after transfection. Cytochalasin D treatment was performed by incubating cells at the room temperature for 2 h in 4 μM cytochalasin D/DMEM/5% fetal bovine serum, which was freshly prepared from 2 mM cytochalasin D/DMSO (dimethyl sulfoxide) stock solution. Cells treated with DMSO (1:500) in DMEM/5% fetal bovine serum under the same condition were used as a negative control for cytochalasin D treatment.

Immunoprecipitation and western blotting

Transfected HEK293 cells were collected from the culture medium, homogenized in lysis buffer (50 mM Tris–HCl pH 8.0, 150 mM NaCl, 0.5% Triton X-100, 5 mM ethylene diamine triacetic acid (EDTA), 0.5 mM phenylmethylsulfonyl fluoride, 1× protease inhibitor and 1 mM DTT) and sonicated briefly. Alternatively, mouse retinas were dissected and homogenized in lysis buffer. Then, the cell or retinal lysate was centrifuged at 18 000g for 10 min, and the supernatant was precleared by incubation with protein G sepharose (Amersham Biosciences) for 1 h. Subsequently, the supernatant was incubated with a primary antibody for 3.5 h and centrifuged at 18 000g for 10 min. The resulting supernatant was incubated with protein G sepharose for 1 h. After a brief centrifugation at 2000g, the pellet was washed with lysis buffer four times and then boiled in Laemmli sample buffer. All the procedures were performed at 4°C. A non-immune rabbit immunoglobulin served as a negative control. Cells without the immunoprecipitated protein or whirlin knockout retinas were included as a second negative control.

For western blotting, two retinas from one mouse or a similar amount of other mouse tissues were homogenized in 100 μl of the RadiolImune Precipitation Assay (RIPA) buffer, sonicated and boiled with the Laemmli buffer for 10 min. The various mouse tissue samples, cell lysates or the above immunoprecipitates were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to a polyvinylidene fluoride (PVDF) membrane. The resulting PVDF membrane was sequentially subjected to blocking for 1 h, primary antibody incubation overnight at 4°C and secondary antibody incubation for 1 h. The protein bands were detected using the chemiluminescent substrate.
with the AlphaInnotech AlphaView software on a FluorChem Q machine (Cell Biosciences, Inc., Santa Clara, CA, USA). The intensities of protein bands were measured by Image J and normalized using sample loading control bands.

**Immunofluorescence staining and colocalization analysis**

Cultured cells were fixed in a mixture of methanol and acetone (1:1) at \(-20^\circ\text{C}\) for 10 min and rinsed with PBS 5 min three times. Encuclued eyes were frozen immediately in liquid nitrogen and sectioned at 10 \(\mu\text{m}\). The obtained retinal sections were fixed in 2\% formaldehyde/PBS for 5 min and permeabilized by 0.2\% Triton X-100/PBS for 5 min. The mouse cochlea at postnatal day 3–5 was dissected, fixed in 4\% formaldehyde/PBS for 10 min and permeabilized by 0.2\% Triton X-100 for 5 min. The fixed cells, retinal sections or cochleae were then blocked in 5\% goat serum/PBS for 1 h, incubated with primary antibodies in 5\% goat serum/PBS at appropriate dilution ratios at 4\(^\circ\text{C}\) overnight, washed several times with PBS and then incubated with the Alexa fluorochrome-conjugated secondary antibodies and Hoechst dye 33342 in 5\% goat serum/PBS for 1 h. For double staining of espin and whirlin in cultured cells, the rabbit espin (K-14) and chicken whirlin antibodies were used. The chicken whirlin antibody did not give good signals in tissue staining. Therefore, to double stain retinal sections and cochleae, the rabbit espin (K-14) and biotin-labeled rabbit whirlin (PDZIE) antibodies were used. The immunostaining procedure was modified as follows. The retinal sections and cochleae were first stained with the rabbit espin antibody and Alexa Fluor\(^{\text{®}}\) 594 goat anti-rabbit secondary antibody as described above. Then, they were incubated with 0.45 mg/ml non-immune rabbit immunoglobulin (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) for 2 h, washed with PBS, incubated with the biotin-labeled rabbit whirlin antibody/5\% goat serum/PBS overnight, washed with PBS and finally incubated with Alexa Fluor\(^{\text{®}}\) 488-streptavidin for 1 h. For other double staining in this study, primary antibodies from different species were used. Alexa Fluor\(^{\text{®}}\) 488 and 594 secondary antibodies were followed. The stained sections were viewed and photographed on a confocal laser scanning microscope (Model FV1000, Olympus, Tokyo, Japan).

To quantitatively analyze the colocalization of two proteins, the confocal double-staining image was imported into Photoshop as two layers. The region of interest was cropped and the resultant images of each protein were saved as individual TIFF files. Then, the TIFF files were opened in Image J and analyzed using the Plugin, JACoP (31). The Pearson’s coefficients and the cytofluorograms (scatter plots) were utilized to assess the existence of colocalization between two proteins. Image J was also employed to quantify the intensity and area of espin signals in the immunofluorescence staining images.

**Fluorescence recovery after photobleaching (FRAP)**

FRAP was carried out using an inverted confocal laser scanning microscope (model FV1000, Olympus) with the FV10-ASW 2.1 software. Cells were grown in complete normal growth medium in glass bottom dishes (Mat Tek Corporation, Ashland, MA, USA) and sealed with parafilms. The bleached regions of cells were defined manually as \(\sim 1\ \mu\text{m}\) wide circles and were bleached for 250 ms using a full laser power. The recovery of fluorescence was monitored using 1\% laser power at intervals of 4 s. The active extension/retraction of filopodia during our FRAP experiments and the complete fluorescence recovery of the bleached GFP-espin at the filopodia and the bleached GFP-actin in the cytoplasm in its single-transfected cells suggested that our photobleaching procedure did not significantly disturb the cell physiology. In the bleached region, the final fluorescence intensity \(F_{\text{final}}\) after recovery was determined by the mean of fluorescence intensities from at least 10 time points on the plateau of the recovery curve. The final recovery percentage of the fluorescence intensity \(F_{\text{F}}\) was calculated using the following formulas, \(F_{\text{F}} = 100\% \times \left( F_{\text{final}} - F_0 \right) / \left( F_{\text{pre}} - F_0 \right)\), where \(F_0\) and \(F_{\text{pre}}\) are the fluorescence intensities immediately after and before the photobleaching, respectively. \(t_{1/2}\) was determined as the time required for the fluorescence intensity to recover to 50\% of \(F_{\text{final}} - F_0\).

**In situ hybridization**

An espin cDNA fragment (1951–2616 bp, NM_207687) shared by all espin isoforms was amplified from the mouse retina by reverse transcription-polymerase chain reaction, cloned into the GEMTeasy vector (Promega, Madison, WI, USA) and confirmed by DNA sequencing. The antisense and sense RNA probes were transcribed and DIG labeled from the linearized espin cDNA plasmid using DIG RNA labeling kit (Roche, Indianapolis, IN, USA). The mouse eye ball was frozen on dry ice and sectioned at 10 \(\mu\text{m}\) in a cryostat. The obtained retinal sections were fixed in 4\% paraformaldehyde/PBS for 20 min, washed in 2\times standard saline citrate (SSC, 0.3 \(\mu\text{M NaCl}/0.03 \text{ M sodium citrate}) three times, treated with 0.2 \(\mu\text{M HCl}\) for 15 min, incubated in 0.25\% acetic anhydride/0.1 \(\mu\text{M triethanolamine hydrochloride, pH 8.0}\) for 5 min twice and pre-hybridized with hybridization buffer at 60\(^\circ\text{C}\) for 2 h. Then the sections were hybridized with 1 \(\mu\text{g/ml}\) DIG-labeled probes in hybridization buffer at 65\(^\circ\text{C}\) overnight. The hybridization buffer contained 50\% formamide, 5\times SSC, 1 mg/ml torula RNA, 100 \(\mu\text{g/ml}\) heparin, 1 Denhardt’s solution (0.02\%) each of bovine serum albumin, Ficoll and polyvinylpyrrolidone), 0.1\% Tween 20, 5 \(\text{nm}\) EDTA and 0.1\% chaps. After hybridization, sections were washed in 2\times SSC for 10 min three times, incubated in 50\% formamide/1\times SSC at 65\(^\circ\text{C}\) for 30 min twice, washed in 2\times SSC for 10 min, incubated in MA BT buffer (0.1 \(\mu\text{M maleic acid, 0.15 M NaCl, 0.1\% Tween 20, pH 7.5}\) for 10 min three times and blocked in 0.5\% blocking reagent (BR)/MA BT for 2 h. Lastly, sections were incubated with anti-DIG-AP fab fragment/BR/MA BT overnight, washed in 2 \(\text{mm levamisole/MA BT}\) for 10 min three times, in AP buffer for 10 min twice (5 \(\text{mm levamisole, 0.3 M NaCl, 50 mm MgCl}_2, 0.1\%\) tween 20, 0.1 \(\text{M Tris-Cl, pH 9.5}\)) and developed in NBT/BCIP. BR, anti-DIG-AP fab fragment, NBT/BCIP were purchased from Roche.

**Scanning electron microscopy**

Mouse inner ears were dissected, fixed in 2\% glutaraldehyde/PBS for 2 h and decalcified in 100 \(\text{mm}\) EDTA for several days. The

---

**Note:** The text is a transcription of the content from the page provided. The formatting and layout have been simplified for readability. The original page contains tables and figures that are not transcribed here. The scientific notation and units are retained as closely as possible. The text is organized to maintain the logical flow and structure of the original document.
cochlea was further dissected, rinsed in PBS and post-fixed by alternative incubations in 0.5% osmium tetroxide, water and 0.3% thiocarbohydryzide. Each step was 2 min long. Then the cochlear tissues were dehydrated in graded ethanol series, critical point dried and sputter coated. Images were taken on a JEOL 7401F field emission scanning electron microscope.

Statistical analysis
Student’s t-tests were conducted using Microsoft Office Excel to compare values between two different groups. A P-value of <0.05 was considered to indicate a significant difference between groups.

ACKNOWLEDGEMENTS
We thank Dr James R. Bartles (Northwestern University) for the valuable suggestions on this manuscript and the gift of the espin antibody and cDNA plasmids, Dr Beat Imhof (University de Geneve) for the GFP-actin plasmid, Dr Jianmin Zhang (University of Utah) for the help with in situ hybridization and Mr Michael Adamian (Harvard Medical School) for the scanning electron microscopy support.

Conflict of Interest statement. None declared.

FUNDING
This work was supported by the National Institutes of Health (R01 EY020853, P30 EY014800); the Hope for Vision; the Foundation Fighting Blindness; the E. Matilda Ziegler Foundation for the Blind, Inc.; the startup package of the Moran Eye Center, University of Utah; and an unrestricted grant from Research to Prevent Blindness, Inc., New York, NY, to the Department of Ophthalmology & Visual Sciences, University of Utah.

REFERENCES


