

Figure S1, related to Figure 1. Changes in cochlear sensory epithelium dimensions from E14 to P0.

(A) Thickness of the cochlear epithelium, measured from basement membrane to the luminal surface on the medial and lateral sides of the sensory region near the base. The epithelium is significantly thinner at P0, compared to E14 and E16 ($p < 0.01$).

(B) Medial to lateral width of the sensory epithelium (organ of Corti, OC) from E14 to P0. The OC is significantly wider at P0 ($p < 0.01$).

(C) Length occupied by 100 prosensory or sensory cells along the base-to-apex axis of the cochlea. All values differ significantly ($p < 0.01$). For (A-C), $n = 5$ samples for each age.

(D) Cellular stratification is present in the mid-apical region of the cochlea at E16. (D) Single-plane yz view of the mid-apical region of the cochlear duct. Developing HCs and SCs are marked by expression of CDKN1B (red) and SOX2 (blue). (D') Same as (D), but with cell nuclei marked by asterisks. Note the three layers of cells. (D'') Orthogonal xz view at the position of the arrow in (D') illustrating the persistence of three or more stratified rows of cells in the OC.

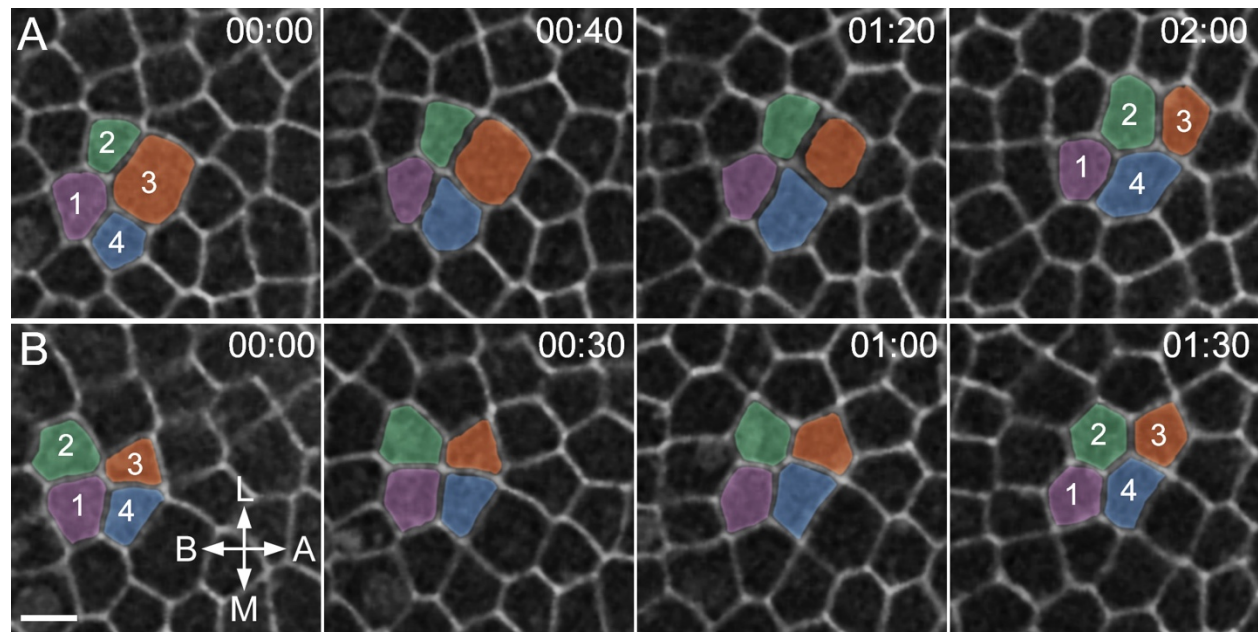


Figure S2, related to Figure 3. Cell intercalation is observed near the luminal surface of the cochlear epithelium.

Individual frames of a 2 μm Z-stack projection taken near the luminal surface of an E15-equivalent *R26R^{mt-mG}* cochlear explant from a 4D time-lapse sequence, with four cells pseudo-colored to aid visualization. Membrane tdTomato is shown in grayscale.

(A) Cell intercalation occurs in the medial-lateral direction over the course of 2 hours in this example.

(B) In this example, the new border between cells 2 and 4 is approximately 45° from the direction of elongation.

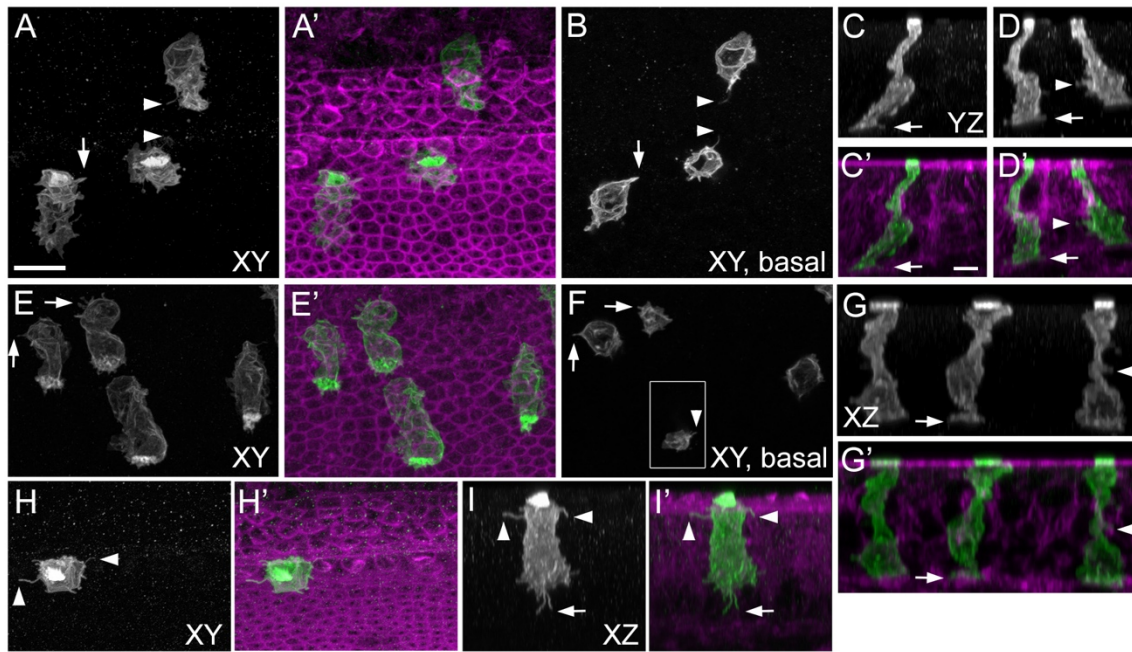


Figure S3, related to Figure 4. Protrusions are present on supporting cells and hair cells in vivo.

(A-C) Images of the mid-base region of the cochlea from an E15 *Sox2^{CreERT2}; R26R^{mT-mG}* embryo. Membrane EGFP is shown in grayscale, followed by a merged image of luminal phalloidin staining (magenta) and mEGFP (green).

(A, A') *xy* projections of a confocal Z-stack through the cochlear epithelium. mEGFP-labeled SCs cells extend lateral protrusions (arrow and arrowheads).

(B) Projection of the basal-most 3 μm of the cells shown in (A) illustrates protrusions located near the basement membrane. Thin, filopodia-like extensions (arrowheads) and wider protrusions (arrow) are visible.

(C, D) *yz* projections of the cells in (A, B), illustrating SC morphologies. The left cell in (A) is shown in (C), and the two cells on the right are shown in (D). Protrusions along the basement membrane (arrows) and lateral protrusions midway between luminal and basal surfaces (arrowhead) are indicated.

(E-G) Labeled SCs/PrCs from the apical region of an E15 cochlea. Protrusions similar to ones illustrated in A-D are present.

(F) Projection of basal-most 3 μm of cells shown in (E). Inset shows a fine protrusion (arrowhead) on the medial cell in E, the basal surface of which was at a different Z level.

(G, G') *xz* projection of the lateral cells shown in (E). Similar protrusions are observed as in (C, D).

(H, H') mEGFP-labeled HC from the base of the cochlea at E15. In contrast to PrCs/SCs, only thin, filopodia-like protrusions are present (arrowheads).

(I, I') *xz* projection of the cell shown in (H). Protrusions are found extending laterally near the luminal surface (arrowheads), and extending toward the basement membrane (arrow). Scale bars, A, 10 μm (same for B, E, F, H, I); C, 5 μm , same for (D, G).

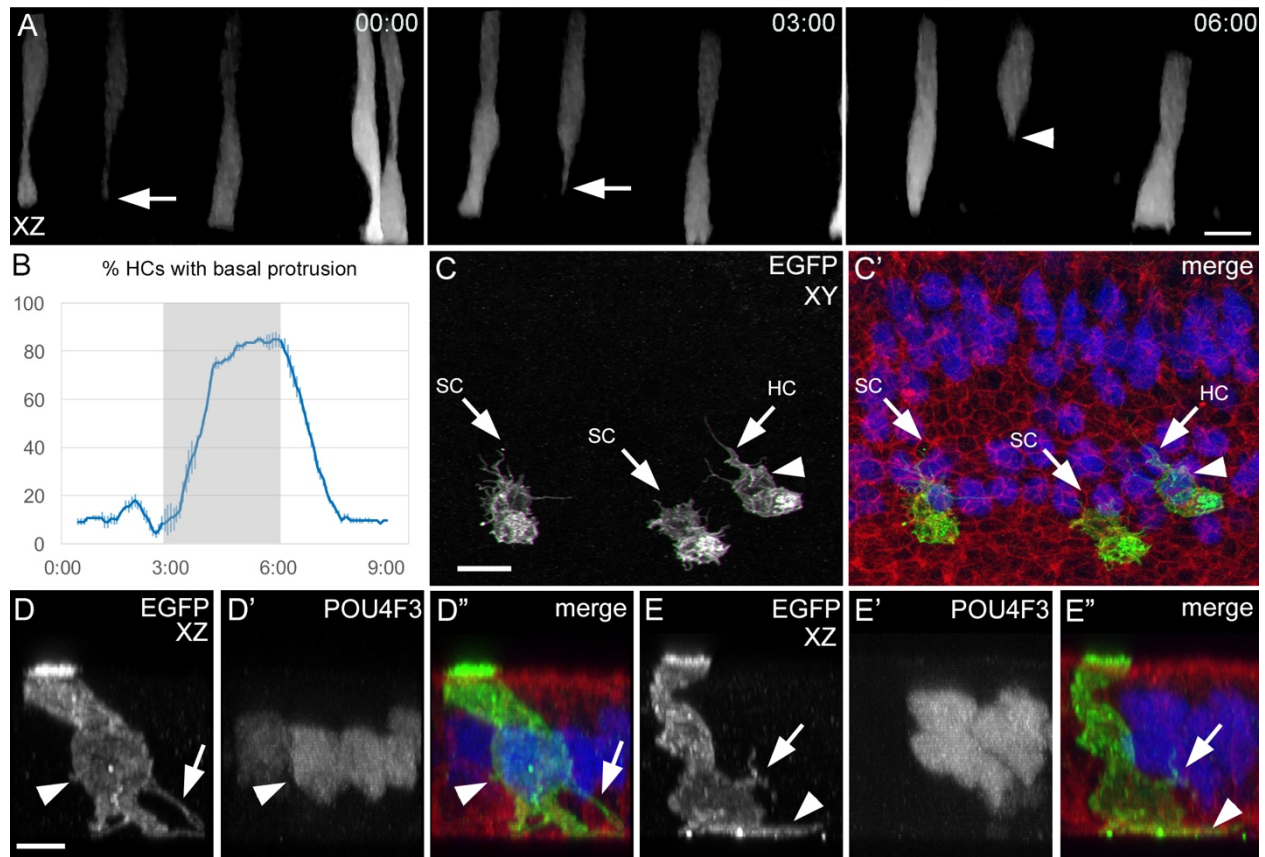


Figure S4, related to Figure 5. Hair cell differentiation is not dependent on Myosin II activity

(A) *xz* projections of a ZsGreen-labeled explant at E14, from Movie 8. At 0:00, PrCs extend from the luminal surface to the basement membrane. One cell has a thinner basal extension (arrow), which no longer contacts the basement membrane by 3:00. By 6:00 this cell has a more mature HC morphology (arrowhead). Scale bar, 10 μ m.

(B) Percentage of HCs extending a basal protrusion during blebbistatin treatment (boxed region). 85% of labeled HCs have a protrusion after 2 hours of 10 μ M blebbistatin exposure, but the protrusions retract when blebbistatin is washed out after 6 hours. $n = 4$ explants, 12 cells.

(C-C') *xy* projection of a Z-stack from an E16 equivalent *Sox2^{CreERT2}; R26R^{mT-mG}* explant treated with 10 μ M blebbistatin for 3 DIV. Three cells are labeled with anti-GFP in C. All three extend numerous thin protrusions (arrows). (C') Merge of C and C' confirms the identity of the labeled cells as one IHC (HC), based on its position and POU4F3-positive nucleus, and two supporting cells (SC). EGFP (green), POU4F3 (blue), and phalloidin (red). Scale bar, 10 μ m.

(D-E'') *xz* projections of individual cells shown in (C), labeled as above. Scale bar, 5 μ m.

(D-D'') IHC shown in (C). The overall cell morphology is consistent with a HC bottle shape (arrowhead), but protrusions from the basal surface of the cell project toward the basement membrane (arrow).

(E-E'') Similar view as in (D-D'') of one of the SCs in (C). Protrusions both along the basement membrane (arrowhead) and at a more luminal level (arrow) are present.

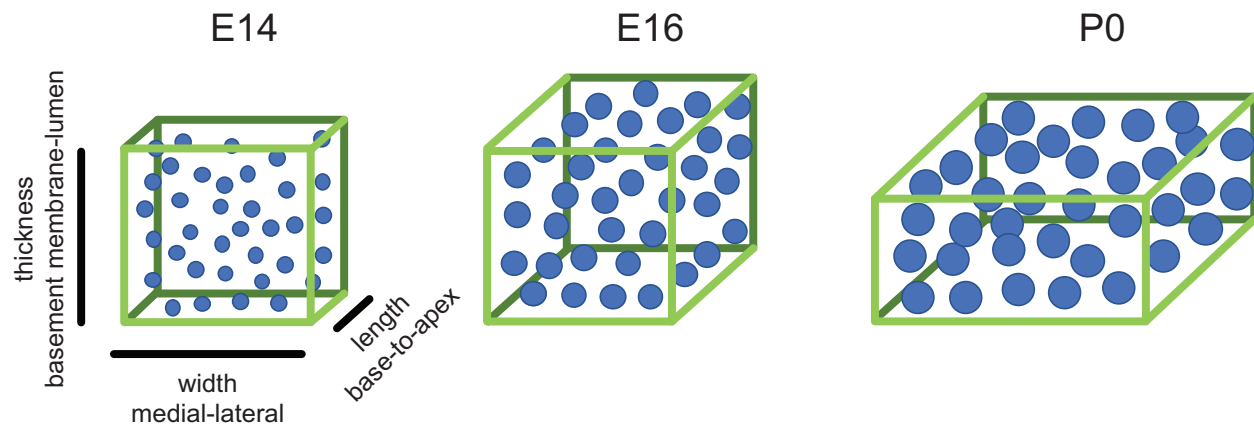


Figure S5. Simple model of cochlear sensory cell redistribution from E14 to P0

Measured values for cells/length or length/cell

E14 = 351/100 μm B-A axis, 35/10 μm B-A axis, 0.285 $\mu\text{m}/\text{cell}$

E16 = 203/100 μm B-A axis, 20/10 μm B-A axis, 0.475 $\mu\text{m}/\text{cell}$

P0 = 173/100 μm B-A axis, 17/10 μm B-A axis, 0.578 $\mu\text{m}/\text{cell}$

E14 B-A length of 100 PrCs = 28.67 μm , 0.286 $\mu\text{m}/\text{cell}$

E16 B-A length of 100 PrCs = 47.59 μm , 0.492 $\mu\text{m}/\text{cell}$

P0 B-A length of 100 PrCs = 61.33 μm , 0.613 $\mu\text{m}/\text{cell}$

Length of entire sensory epithelium, E14: 2196.9 μm

Length of entire sensory epithelium, P0: 4855.5 μm

At E14 the PrCs along a 10 μm length of the B-A axis = 35. At E16, a similar 10 μm block of epithelium contains only 20 PrCs, suggesting that the other 15 cells have been redistributed through intercalation and/or migration. The increased length that results from the redistribution of these cells, assuming no change in cell volume, can be estimated using the measurements for the length along the B-A axis of 100 PrCs at E14, which is 28.67 μm . Therefore, 15 cells would generate an extension of $28.67/100 \times 15 = 4.30 \mu\text{m}$. Therefore, the total distance covered by the original 35 PrCs based on redistribution = 14.30 μm (10 + 4.30). But, in addition to redistribution, the cells have increased in volume. Since this model does not make a distinction between HCs and SCs and describes extension along the B-A axis, the easiest way to estimate the contribution to extension as a result of change in cell volume is to compare the distance covered along the B-A axis by 100 cells between E14 and E16. At E14, this value is 28.66 μm ; at E16, the value is 47.59 μm , an increase of 166%. Assuming this increase is uniform over every cell, then $14.30 \mu\text{m} \times 1.66 = 23.72 \mu\text{m}$. Therefore, the estimated total extension along the B-A axis between E14 and E16 would be $23.72/10 = 237\%$.

The same calculation can be made between E16 and P0. Based on the basal-apical distance covered by 100 cells at E16, 47.59 μm , individual cell length is 0.48. Between E16 and P0, the decrease in cells along 100 μm of the basal-apical axis is 30 cells. Therefore, for our original 35 cells, we assume that 3 have migrated along the basal-apical axis leading to an increased extension, based on cell migration of 1.41 μm . There is also an increase in cell extension along the B-A axis for 100 cells to 61.33 μm at P0. $61.33/47.59$ (cell size at E16) = 129% increase. Therefore, the full extension between E16 and P0 would be 23.27 (extension at E16) + 1.41 (migration) \times 1.29 (extension along B-A axis from change in cell volume) = 31.84 μm . And total extension between E14 and P0 would be 10 μm to 31.84 μm , an increase of 318%. By comparison, measured extension of the entire cochlear sensory epithelium between E14 and P0 = $4855.5 \mu\text{m}$ (length @P0)/ $2196.9 \mu\text{m}$ (length @E14) \times 100 = 221%. Possible explanations for this discrepancy are discussed in the text.

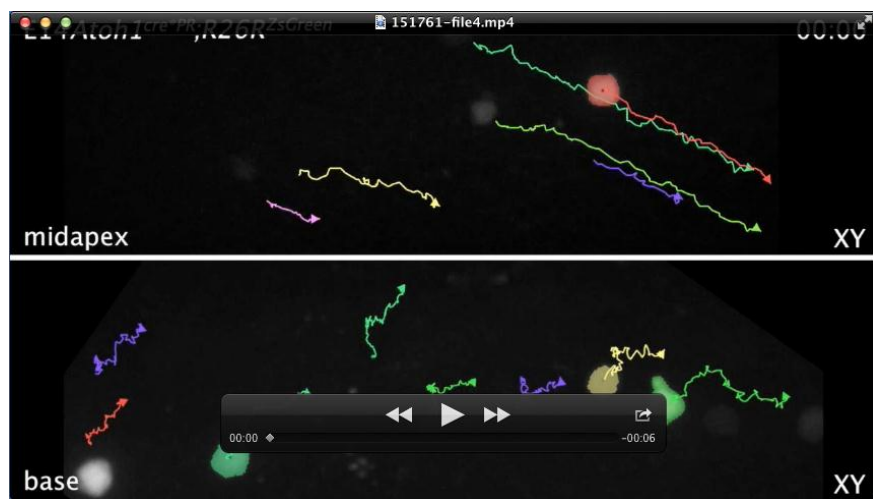
Supplemental movies.

For all movies, the apical direction of the cochlea is to the right of the image. The medial (IHC) side of the sensory epithelium is towards the bottom, and lateral (OHC) side is to the top. Some images have been flipped horizontally for consistency in presentation. All time-lapse sequences are replayed at 15 frames per second (fps), and the capture rate is indicated in HH:MM in the counter within the image and the figure legend.



Movie 1, related to Figure 2. Cells in the sensory epithelium of cochlear explants move toward apex.

Low magnification time-lapse of a full Z-stack projection from an *Atoh1*^{Cre*PR}; *R26R*^{tdTomato} cochlear explant established at E13 and imaged for 15 hours beginning at 2 DIV (E15 equivalent). Lengthening and slight narrowing of the population of labeled cells is apparent as cells move in the apical direction. Images were captured at 6 min. intervals.



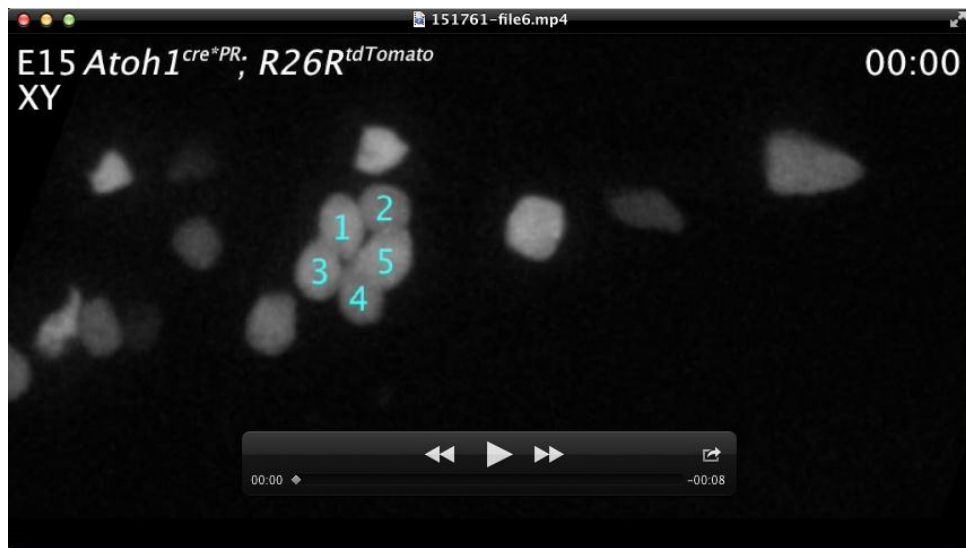
Movie 2, related to Figure 2. Displacement of apical cells is greater and more directed than that of basal cells.

15-hour time-lapse movie of XY projections of confocal Z-stacks taken near the mid-apex (top) and base (bottom) of an E14 equivalent *Atoh1*^{Cre*PR}; *R26R*^{ZsGreen} explant. Individual cells and migration tracks are pseudo-colored. Images were captured at 10 min. intervals. Some cells become brighter and visible within the imaged region during the time-lapse as ZsGreen accumulates.



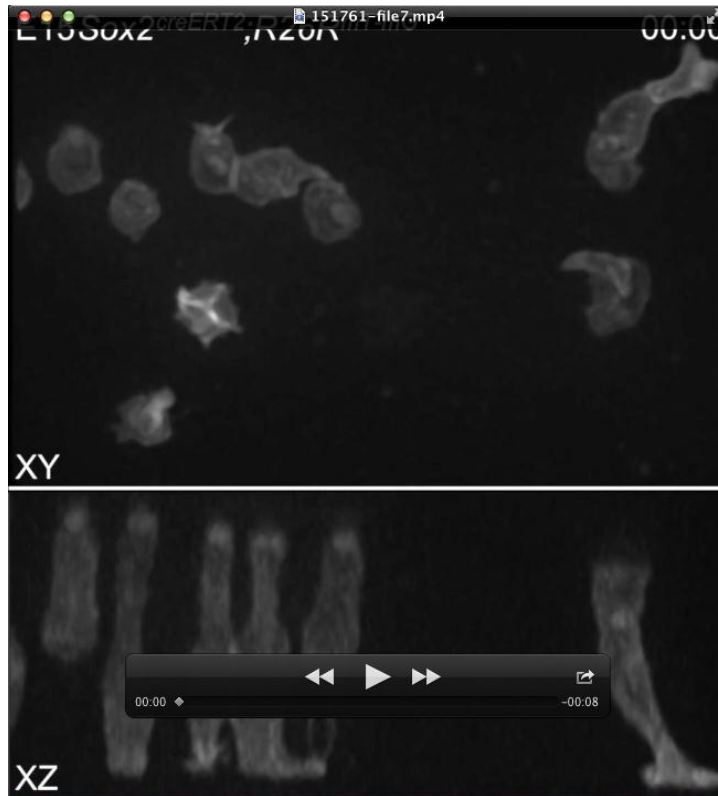
Movie 3, related to Figure 3. Evidence of convergence and extension in E14 cochlear explant.

XY (top) and XZ (bottom) views of a 16-hour time-lapse sequence of the mid-apical region of an E14 *Atoh1*^{Cre*PR}; *R26R*^{ZsGreen} explant (same explant as in Movie 2). Cells become further apart along the extension (X) axis, while converging along the Y-axis. Images were captured at 10 min. intervals.



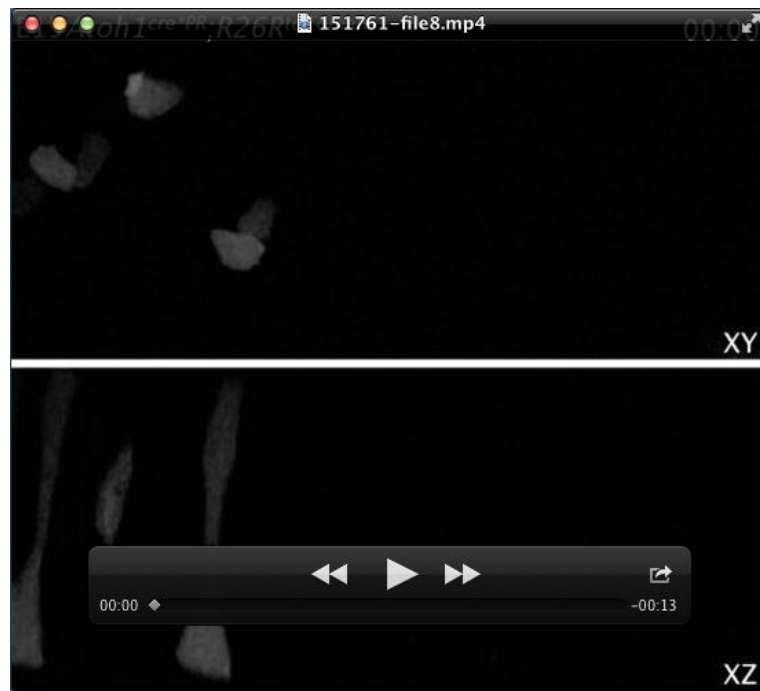
Movie 4, related to Figure 3. HCs align into rows in an E15 cochlear explant.

8-hour time-lapse movie from an E15-equivalent *Atoh1*^{Cre*PR}; *R26R*^{tdTomato} cochlear explant. Presumptive HCs initially in a rosette (labeled in frame 1) resolve into 1st and 2nd row OHCs over the course of 6 hours. Images were captured at 4 min. intervals.



Movie 5, related to Figure 4. Both HCs and SCs exhibit cellular protrusions.

Considerable protrusive activity of both HCs and SCs is apparent in a *Sox2^{CreERT2}; R26R^{mT-mG}* explant imaged at E15. mEGFP in scattered cells is shown in grayscale. XY (top) and XZ (bottom) projections of confocal Z-stacks of a 10-hour time-lapse sequence. Images were captured at 5 min. intervals.



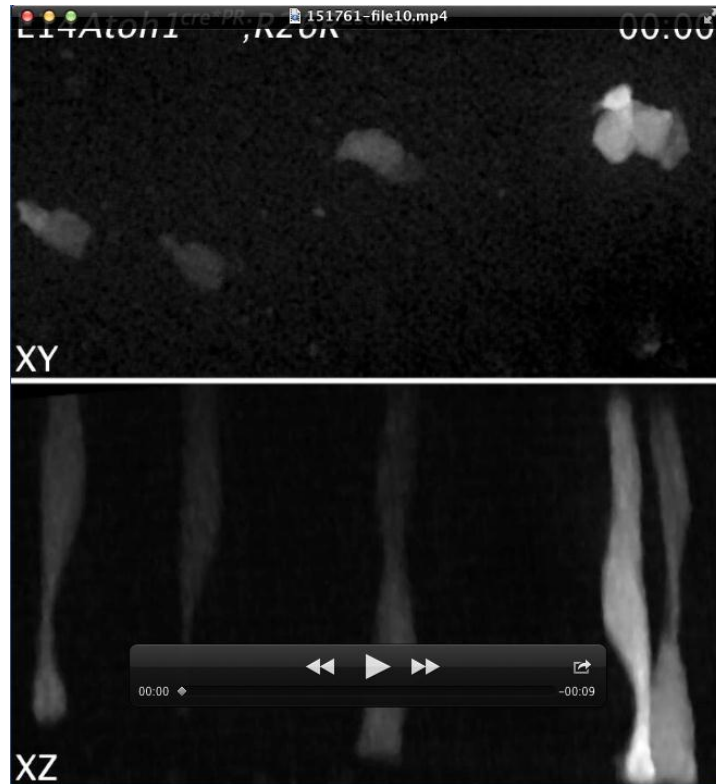
Movie 6, related to Figure 5. Cell movement stalls and HCs extend basal protrusions during MyoII inhibition.

XY (top) and XZ (bottom) views of a 10-hour time-lapse movie from an E15 *Atoh1^{Cre*PR}; R26R^{tdTomato}* explant (two image sequences are spliced together at the 06:15 mark, but all images are of the same explant). 10 μ M blebbistatin was added after 3 hours, and diluted to 1 μ M at 6 hours. The movement of both SCs and HCs in the apical direction stalls during while MyoII activity is inhibited by blebbistatin treatment, and cells extend numerous thin protrusions, including a basally-directed protrusion from presumptive HC. Images were acquired at 3 min. intervals.



Movie 7, related to Figure 5. Blebbistatin disrupts cell movement and patterning.

4-hour time-lapse movie from an *Atoh1*^{Cre*PR}; *R26R*^{tdTomato/mT-mG} E16 explant labeled with both membrane tdTomato and sparse Cre-induced cytoplasmic tdTomato expression to indicate the position of the sensory epithelium. Myosin II activity is inhibited with 10 μ M blebbistatin for 90 mins., beginning at 2 hours. Movement in the apical direction stops during blebbistatin treatment, but resumes after wash-out, though the organization of the epithelium is still somewhat disrupted. Images were acquired at 3 min. intervals.



Movie 8, related to Figure S4. Basal protrusions of PrCs retract quickly during HC differentiation.

Z-stack projections of 6-hour time-lapse sequence of an E14 *Atoh1*^{Cre*PR}, *R26R*^{ZsGreen} cochlear explant, shown in XY (top) and XZ (bottom) views. PrCs spanning from the basement membrane to the luminal surface move toward the apex. One PrC, initially in contact with the basement membrane, retracts its basal protrusion (arrow) from about 04:30 to 06:00, assuming a more HC-like shape (arrowhead). Images were acquired at 5 min. intervals.