Developmental Cell *16*

Supplemental Data

Hey2 Regulation by FGF Provides a *Notch*-Independent Mechanism for Maintaining Pillar Cell Fate in the Organ of Corti

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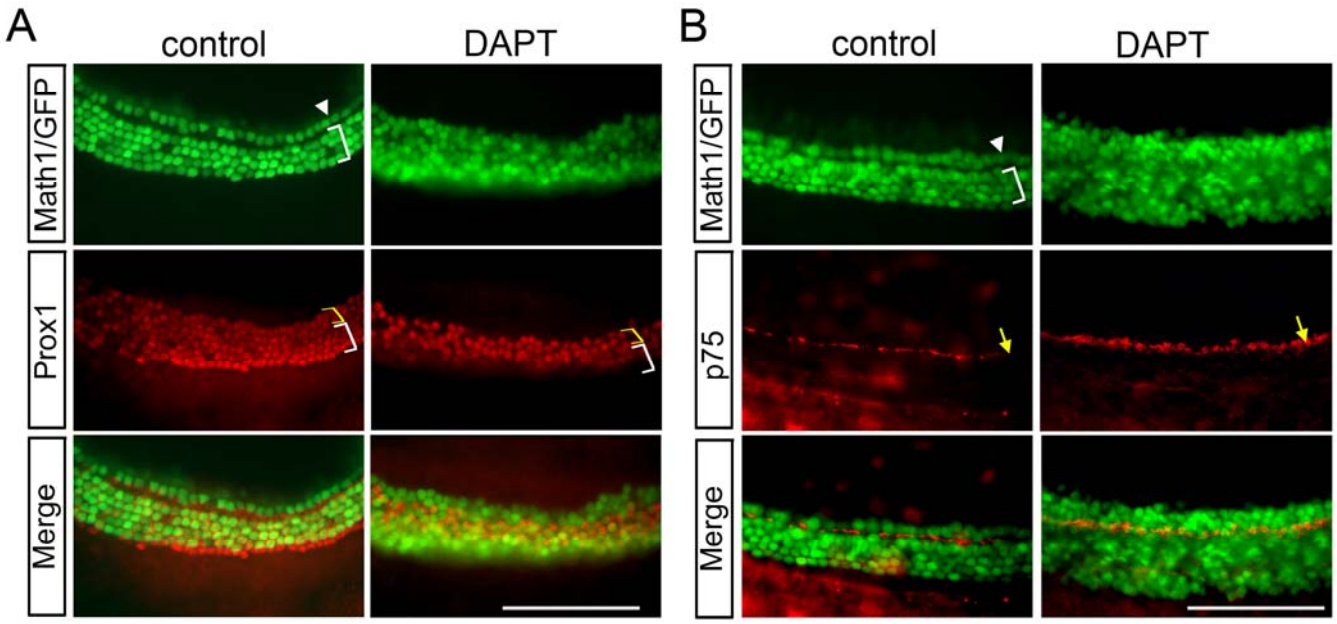

Figure S1. Embryonic (E14.5) Pillar Cells Differentiate in the Absence of Notch Signaling
(A and B) *Math1*/GFP transgenic embryonic day 14.5 (E14.5) cochlear explants were placed in culture for 24 hours and then kept for 48 hours in control (DMSO) or experimental (3 μM DAPT) conditions.
(A) Response of progenitor cells to loss of Notch signaling. DAPT treatment results in an overproduction of hair cells (*Math1*/GFP) in both the inner (arrowhead) and outer (bracket) hair cell region, as well as a reduction in the number of Prox1+ cells (red) in the outer hair cell region (white bracket). However, Prox1+ cells in the pillar cell region (yellow bracket) persist.
(B) Presence of pillar cell specific p75 staining (red) in control (control, yellow arrowhead and DAPT treated cochlear explants (DAPT, yellow arrowhead) indicates that pillar cell differentiation is not affected by loss of Notch signaling. Scale bar: 50μm in A and B.

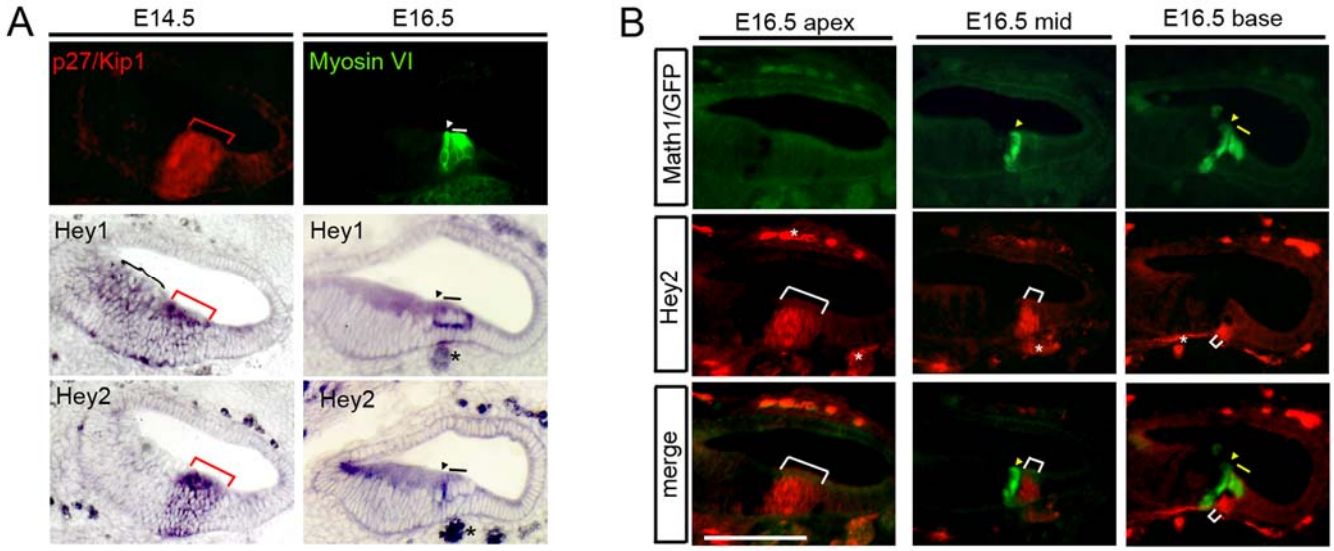

Figure S2. Expression Pattern of Hey1 and Hey2 in Developing Organ of Corti

(A) Prior to hair cell differentiation (E14.5), *Hey1* and *Hey2* are expressed in the pro-sensory domain progenitor cells. Adjacent E14.5 cochlea sections were stained with $p27^{Kip1}$ antibody to mark the pro-sensory domain (red) and hybridized with *Hey1* and *Hey2* riboprobes. Red bracket marks the $p27^{Kip1}$+ pro-sensory domain. Note *Hey1* expression domain extends into future Köllikers organ (brace). During hair cell differentiation (E16.5), *Hey1* and *Hey2* expression becomes restricted to future pillar and Deiters' cells region. Adjacent E16.5 cochlea sections were stained with Myosin VI antibody to mark differentiating hair cells (green) and hybridized with *Hey1* and *Hey2* riboprobes. Black arrowhead points to inner hair cell, black bar marks outer hair cell region.

(B) E16.5 Math1/GFP transgenic cochlear sections were stained with Hey2 antibody (red). Native Math1/GFP expression (green) marks inner (yellow arrowhead) and outer hair cells (yellow bar). White bracket marks Hey2 expression domain. Following the basal to apical gradient of hair cell differentiation (Math1/GFP, green), Hey2 protein (red) becomes progressively restricted to future pillar cell nuclei (base, white bracket). Note * marks blood vessel. Scale bar: 100 μm in A, B.

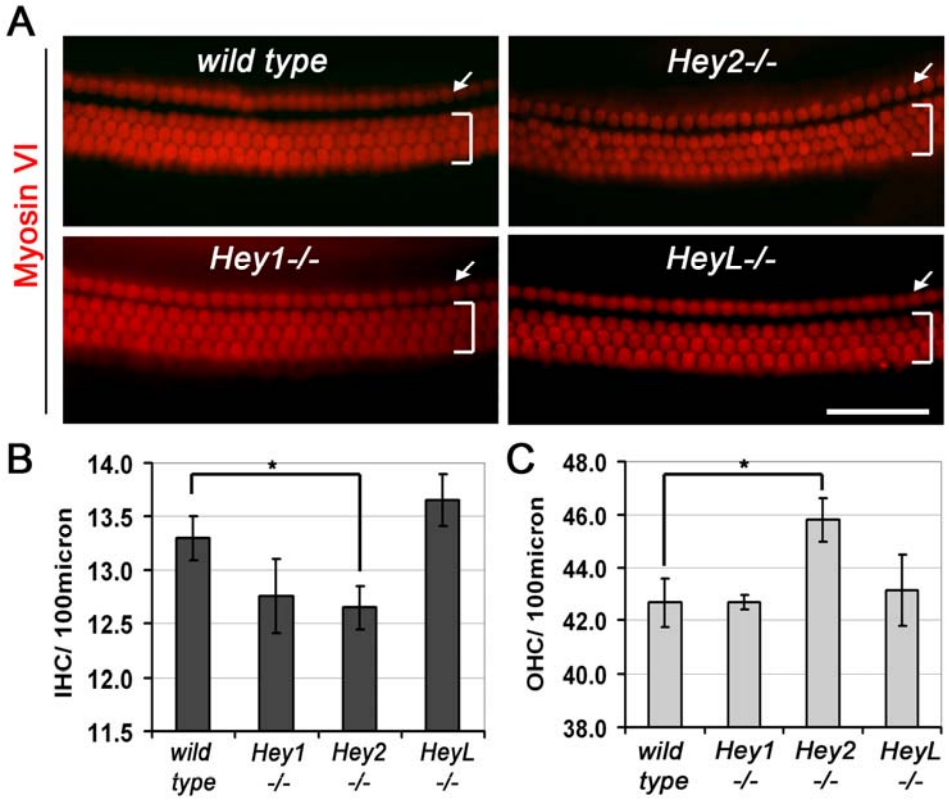

Figure S3. *Hey2* Loss of Function Results in Mild Overproduction of Outer Hair Cells
(A) Cochlear whole mounts were prepared from wild-type, *Hey1*-/-, *Hey2*-/-, and *HeyL*-/- newborn mice (P0) and stained for hair cell marker Myosin VI (red). Mid-turn segments are shown. Arrow points to row of inner hair cells, bracket marks three rows of outer hair cells.
(B and C) Quantification of inner (B) and outer hair cell (C) density of the mutant and wild-type cochlear whole mounts. Inner hair cells (IHC) and outer hair cells (OHC) were counted along the entire length of cochlear duct. Total length of cochlear duct was measured and used to normalize total number of IHC and OHC to a length interval of 100µm. For each genotype three independent cochlear whole mounts were analyzed. (*P<0.05) (error bars ± s.e.m.). Scale bar: 50 µm in A.

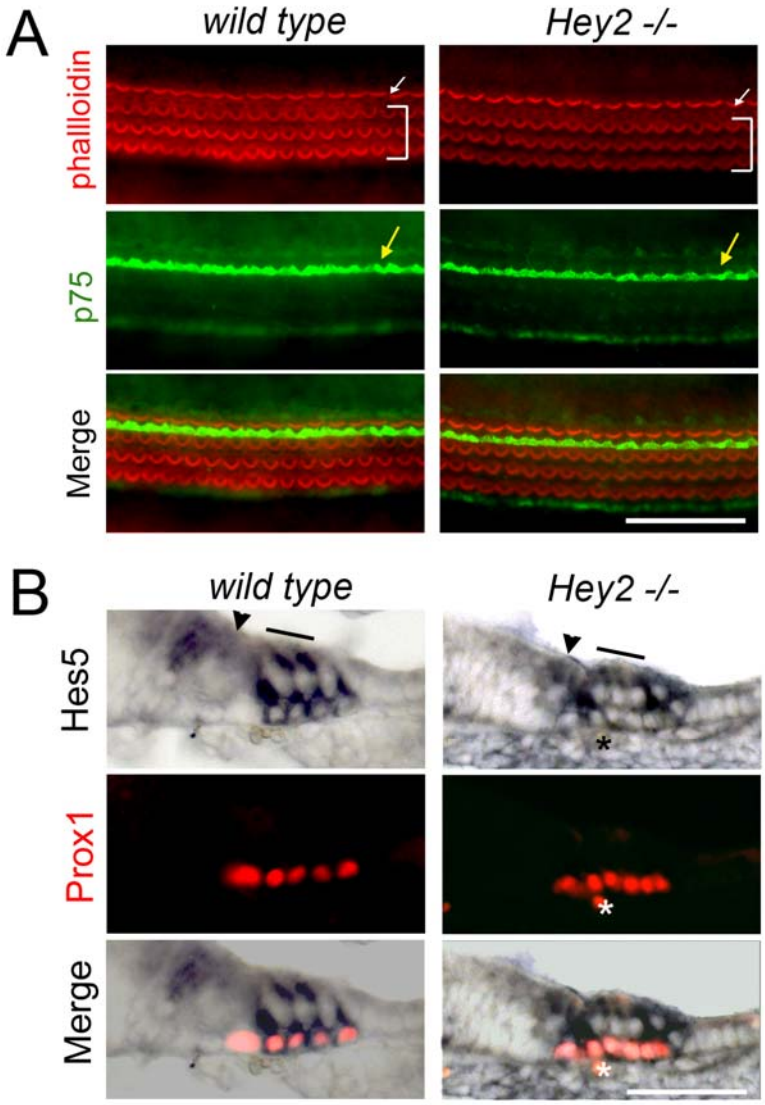

Figure S4. *Hes5* Compensates for the Loss *Hey2* Function in Pillar Cells
(A) Normal pillar cell phenotype in neonatal *Hey2* mutant cochlea (*Hey2*-/-). P75 (green) marks apical surface of pillar cells, actin-rich hair cell stereocilia are visualized using phalloidin staining (red).
(B) In the absence of *Hey2*, *Hes5* expression domain is expanded into pillar cell region and Hensen cell region. P0 wild-type and *Hey2* -/-cochlea section were hybridized with *Hes5* riboprobe and co-labeled with Prox1 antibody staining (red) and *Hes5* in situ. * Debris causing auto-fluorescence. Scale bar 50 µm in A, B.

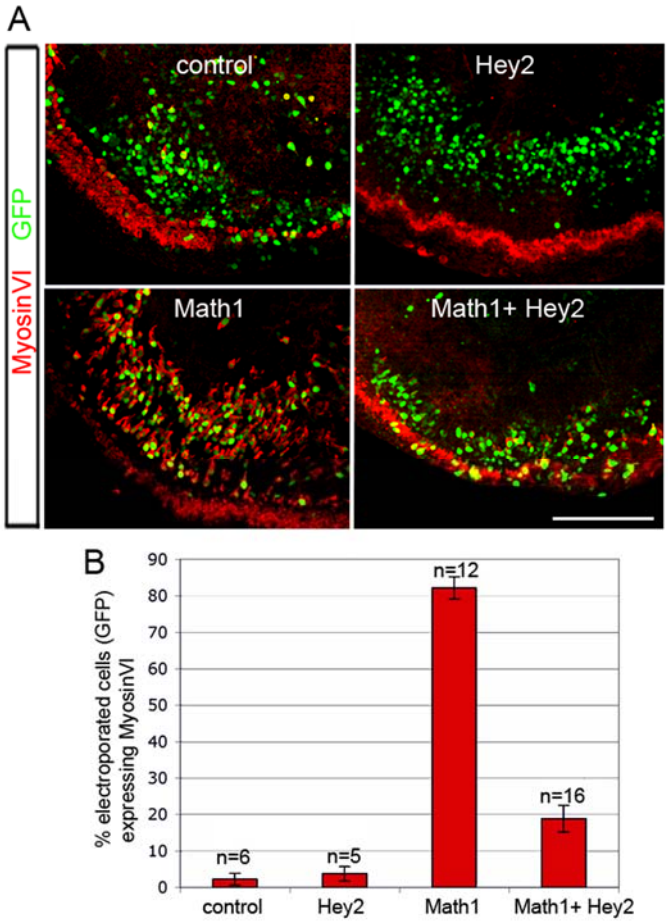

Figure S5. *Hey2* Represses Hair Cell Generation in the Organ of Corti
(A) *Hey2* inhibits *Math1*-induced ectopic hair cell production. E13.5 cochlea were transfected with empty vector (control), *Hey2* (*Hey2*), *Math1* (*Math1*) or *Math1* and *Hey2* (*Math1+Hey2*) expression constructs. To identify transfected cells, a GFP expression plasmid was co-transfected (green, nucleus). After 2 days in culture, staining with hair cell marker MyosinVI (red) identifies hair cells which are either endogenous (GFP-) or ectopic (GFP+). Green and red fluorescent images were captured using confocal microscope.
(B) Quantification of A. Graph shows the percentage of transfected cells (GFP+) which produced hair cells (MyosinVI +/ GFP+) for each condition. A minimum of 5 transfected cochlear organs were analyzed for each condition (error bars ± s.e.m.). Scale bar 100 μm in A.

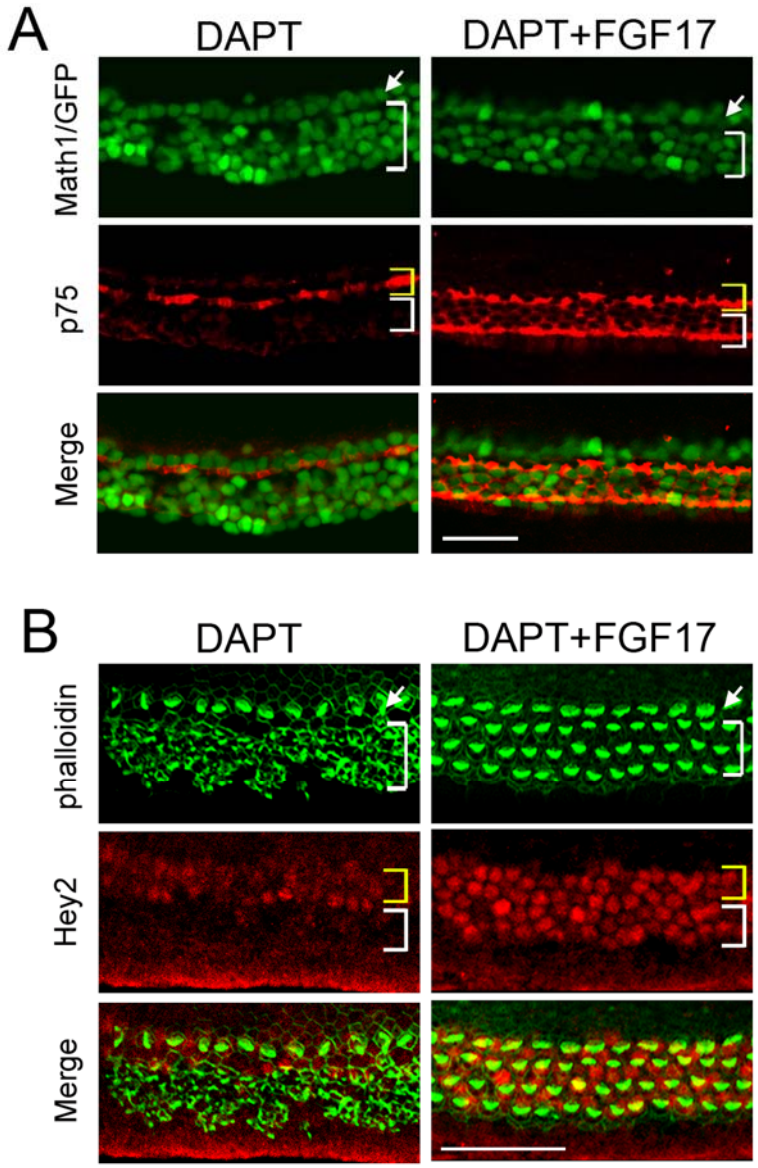

Figure S6. Up-Regulation of Hey2 Expression in Deiters' Cells by FGF17 Prevents Transdifferentiation in the Absence of Notch Signaling

(A) As seen in cochlear explants treated with FGF17 alone (see Fig. 6) 48 hours of FGF17 and DAPT treatment expands pillar cell specific p75 expression (red, yellow arrow) to Deiters' cells (red, white bracket). *Math1*/GFP expression (green) marks inner (white arrow) and outer hair cells (white bracket).

(B) FGF17 up-regulates Hey2 expression in Deiters' cells in the absence of Notch signaling (FGF17+DAPT, white bracket). Phalloidin staining (green) labels the actin rich inner (arrow) and outer hair cell bundles (bracket). Scale bar 50 μm in A and B.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Genotyping Primers

Pax2-Cre

Cre1F: 5'-gcctgcattaccggtcgatgcaacga-3'

Cre1R: 5'-gtggcagatggcgcggcaacaccatt-3'

RBP-J Floxed, Deleted and Wild-Type Allele

RBPJ1: 5'-aacatccacagcaggcaa-3'

RBPJ2: 5'-gatagaccttggtttgtttgg-3'

RBPJ3: 5'-ccactgttgtgaactggcgtgg-3'

Note: These yield a 500bp band for the floxed allele, a 700bp band for the deleted allele and a 300bp band for the wild type allele.

Notch1 Floxed, Deleted and Wild-Type Allele

MBTP71: 5'-acccttgcctcagttcaaacacaagatacg-3'

MBTP01b: 5'-tggcctgcctgtctggaacaacagttcagg-3'

MBTP02b: 5'-actgtcagtcaagccagttcagggcacatgg-3'

Note: MBTP71 and MBTP01b yield a 520bp band for the deleted allele. MBTP01b and MBTP02b yield a 510bp band for floxed and a 440bp band for wild type allele.

Gene–Specific Primers Used for Q-PCR

Gene	Forward	Reverse
Math1 (Atoh1):	5'-atgcacgggctgaacca-3'	5'-tcgttgttgaaggacgggata-3'
L19 (RpL19)	5'-ggtctggttggatcccaatg-3'	5'-cccgggaatggacagtca-3'
Hes5	5'-gcaccagcccaactccaa-3	5'-ggcgaaggctttgctgtgt-3'
Hey1	5'-cactgcaggagggaaaggttat-3'	5'-ccccaaactccgatagtccat-3'
Hey2	5'-aagcgcccttgtgaggaaa-3'	5'-tcgctccccacgtcgat-3'
HeyL	5'-gcgcagagggatcatagagaa-3'	5'-tcgcaattcagaaaggctactg-3'
Hes1	5'-gcttcagcgagtgcatgaac-3'	5'-cggtgttaacgccctcaca-3'
Prox1	5'-cgttacgggagtttttcaatgc-3'	5'-ccttgtaaatggccttcttcca-3'
E-cadherin (Cdh1)	5'-tgtgggtcaggaaatcacatctt-3'	5'-ccaaatccgatacgtgatcttct-3'