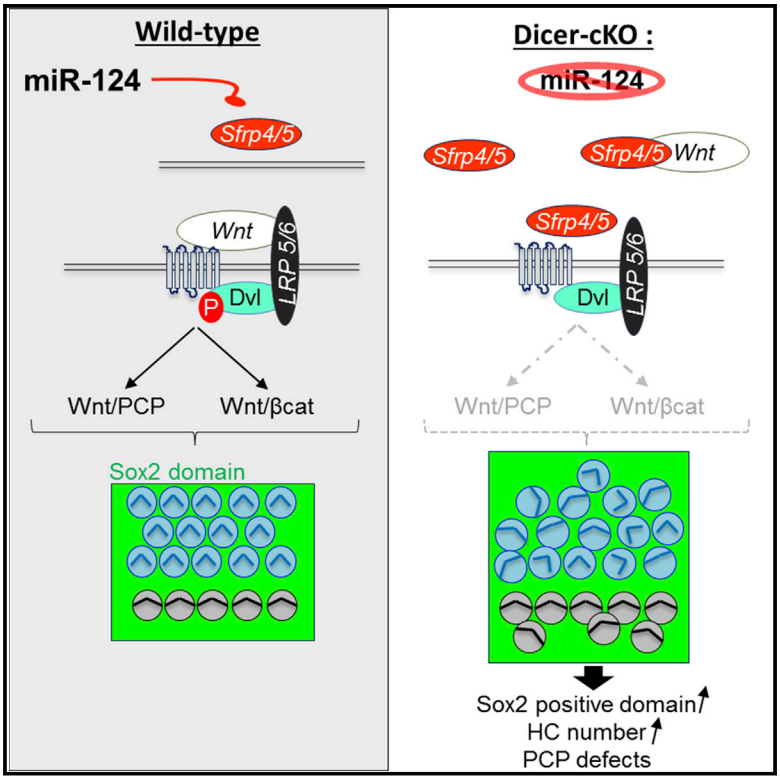
Report

Cell Reports

MicroRNA-124 Regulates Cell Specification in the Cochlea through Modulation of Sfrp4/5

Graphical Abstract



Highlights

- MicroRNAs control cell differentiation and progenitor cell proliferation in the cochlea
- Mir-124 is a regulator of HC differentiation and polarization in the organ of Corti
- MiR-124 targets Sfrp4/5 and modulates both canonical and PCP Wnt signaling pathway

Authors

Aurélia Huyghe,
Priscilla Van den Ackerveken,
Rosalie Sacheli, ..., Laurence Delacroix,
Laurent Nguyen, Brigitte Malgrange

Correspondence

bmalgrange@ulg.ac.be

In Brief

Huyghe et al. show that miR-124 targets secreted frizzled-related protein 4 (Sfrp4) and Sfrp5, two inhibitors of the Wnt pathway, thus feeding into the β-catenin-dependent and also the PCP-related non-canonical Wnt pathways that contribute to hair cell differentiation and polarization in the organ of Corti.

Huyghe et al., 2015, Cell Reports *13*, 31–42
October 6, 2015
http://dx.doi.org/10.1016/j.celrep.2015.08.054

CellPress

Cell Reports
Report

MicroRNA-124 Regulates Cell Specification in the Cochlea through Modulation of Sfrp4/5

Aurélia Huyghe,[1,2] Priscilla Van den Ackerveken,[1,2] Rosalie Sacheli,[1] Pierre-Paul Prévot,[1] Nicolas Thelen,[1] Justine Renauld,[1] Marc Thiry,[1] Laurence Delacroix,[1] Laurent Nguyen,[1] and Brigitte Malgrange[1,*]
[1]GIGA-Neurosciences, University of Liege, C.H.U. Sart Tilman, Liege 4000, Belgium
[2]Co-first author
*Correspondence: bmalgrange@ulg.ac.be
http://dx.doi.org/10.1016/j.celrep.2015.08.054

SUMMARY

The organ of Corti, the auditory organ of the mammalian inner ear, contains sensory hair cells and supporting cells that arise from a common sensory progenitor. The molecular bases allowing the specification of these progenitors remain elusive. In the present study, by combining microarray analyses with conditional deletion of *Dicer* in the developing inner ear, we identified that miR-124 controls cell fate in the developing organ of Corti. By targeting secreted frizzled-related protein 4 (Sfrp4) and Sfrp5, two inhibitors of the Wnt pathway, we showed that miR-124 controls the β-catenin-dependent and also the PCP-related non-canonical Wnt pathways that contribute to HC differentiation and polarization in the organ of Corti. Thus, our work emphasizes the importance of miR-124 as an epigenetic safeguard that fine-tunes the expression of genes critical for cell patterning during cochlear differentiation.

INTRODUCTION

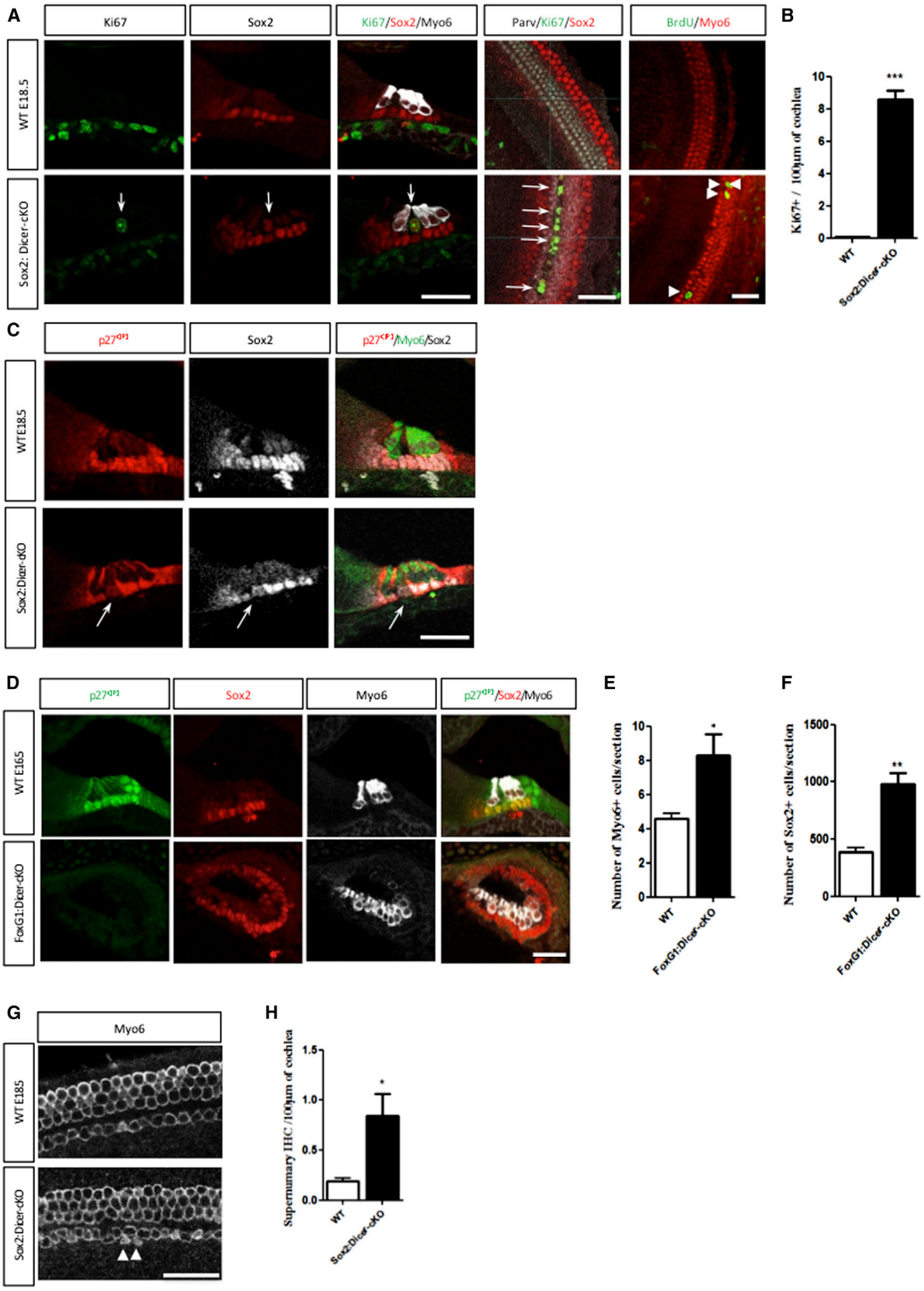
The sensory region of the cochlea—the organ of Corti (OC)—is an epithelial auditory receptor mainly composed of sensory cells, namely, the hair cells (HCs), and distinct types of supporting cells (SCs). These cell types develop from common embryonic progenitors located in the prosensory domain of the otic vesicle and are specialized through activation of specific genetic programs (Groves et al., 2013). HCs are mechanosensory cells that detect sound and convert it into an electrical signal, which is transmitted to the brain by spiral ganglion neurons. In each developing HC, a single microtubule-based kinocilium is positioned at the tip of a V-shaped stereocilia bundle. The appropriate positioning of the kinocilium relative to the stereocilia underlies HC function and is regulated by planar cell polarity (PCP) signaling (Wallingford, 2012). After their specification, HCs organize in three rows of outer HCs (OHCs) and a single row of inner HCs (IHCs).

In mammals, damaged cochlear HCs cannot regenerate, thus their loss leads to irreversible neurosensory deafness. Loss- and gain-of-function studies of genes expressed in the inner ear during development have demonstrated that SCs have a regenerative potential (Sinkkonen et al., 2011). Therefore, defining the cellular and molecular programs that pattern the cochlea and specify HC and SC fate from progenitors will likely contribute to the establishment of new strategies for treating neurosensory deafness.

The differentiation of HCs starts from the prosensory epithelium, at E14.5 in mice, and requires the expression of transcription factors, such as Atoh1 (Bermingham et al., 1999) and signaling cascades, such as the Notch and Wnt pathways (Shi et al., 2014; Zine et al., 2000). Besides these pathways, microRNAs (miRNAs) also contribute to proper cochlear development and HCs maintenance (Friedman et al., 2009; Weston et al., 2011). miRNAs are endogenous non-coding RNAs of ≈22 nucleotides that regulate gene expression through sequence-specific base-pairing with target mRNAs (Bartel, 2004). Many miRNAs have been detected in the developing and mature inner ear (Friedman et al., 2009; Weston et al., 2006), among which some show dynamic expression that correlates with the differentiation of OC (Sacheli et al., 2009). Early embryonic (E8.5) ablation of *Dicer*, an endoribonuclease necessary for miRNA production, in the otic placode using a *FoxG1-Cre* or a *Pax2-Cre* driver line supports a role for miRNA processing in inner ear morphogenesis and innervation (Kersigo et al., 2011; Soukup et al., 2009). Ablation of *Dicer* during HCs differentiation at E14.5 using *Pou4f3-Cre* or *Atoh1-Cre* driver lines results in postnatal malformation of HCs (Friedman et al., 2009; Weston et al., 2011).

How individual miRNAs contribute to these phenotypes is becoming clearer. Members of the miR-183 family are important for promoting cell fate and development of the cochlea (Kuhn et al., 2011; Li and Fekete, 2010). Furthermore, mutations in miR-96 are responsible for non-syndromic progressive hearing loss in mice and humans (Lewis et al., 2009; Mencía et al., 2009).

In the present study, by combining microarray analyses with the conditional deletion of *Dicer* in the developing inner ear, we identify that miR-124 controls cell fate in the developing OC. We show that, by targeting secreted frizzled-related protein 4 (Sfrp4) and Sfrp5, two inhibitors of the Wnt pathway, miR-124 controls β-catenin-dependent and also PCP-related non-canonical Wnt pathways, which contributes to HC differentiation and polarization in the OC.



(legend on next page)

RESULTS

Dicer Controls Proliferation and Differentiation in the Prosensory Epithelium

Previous studies have demonstrated the importance of Dicer during early cochlear development (Soukup et al., 2009). To extend this investigation to the establishment and differentiation of the prosensory domain, we crossed Dicer$^{flox/flox}$ mice (Cobb et al., 2005) with FoxG1$^{Cre/+}$ mice (Hébert and McConnell, 2000) to generate embryos (i.e., FoxG1:Dicer-cKO) with an early (E8.5) depletion of Dicer in the otocyst. To examine whether miRNAs control the establishment of the prosensory domain, we assessed the expression of Sox2 in E14.5 wild-type (WT) or FoxG1:Dicer-cKO cochleae. At this developmental stage, the prosensory Sox2-positive cells have exited the cell cycle to form a zone of non-proliferating cells (Ruben, 1967). Although the size of the cochlea was reduced (data not shown), the Sox2-positive prosensory domain was clearly enlarged in FoxG1:Dicer-cKO animals compared to WT (Figures S1A and S1B). To determine whether this enlarged Sox2-positive cell population in Foxg1:Dicer-cKO embryos resulted from an increased proliferation, we co-labeled these cells with Ki67. In contrast to WT animals, cochlear ducts from FoxG1:Dicer-cKO mice exhibited a significant proliferation in the Sox2-positive cell population (Figures S1A and S1C). In order to avoid the broad defects in organogenesis caused by the early depletion of miRNAs, we then used a Sox2$^{CreERT2/+}$ (Arnold et al., 2011) mouse line allowing for a time-controlled deletion of Dicer. We assessed cell proliferation in cochlear sections from E18.5 embryos that received tamoxifen after the establishment of prosensory cells, at E12.5 and E13.5. The analyses of Sox2:Dicer-cKO cochleae revealed the presence of Ki67-positive cells in the OC (Figures 1A and 1B). Interestingly, all the proliferating cells were Sox2-positive and located between IHC and OHC in the pillar cells region (Figure 1A). Proliferating cells within the OC of Sox2:Dicer-cKO animals were also detected following a single pulse of 5-bromo-2′-deoxyuridine (BrdU) at E18.5 (Figure 1A, white arrowhead).

It is well established that the expression of the cyclin-dependent kinase inhibitor p27^{Kip1} controls the proper timing of cell-cycle exit during prosensory region formation and ensures the quiescence of SCs (Löwenheim et al., 1999). Thus, we checked whether the increased cell proliferation observed in the OC upon Dicer deletion was associated with a reduced expression of p27^{Kip1}. In contrast to WT animals, cochlear ducts from FoxG1:Dicer-cKO mice did not contain any p27^{Kip1}-positive cells at E14.5 and E16.5 (Figures S1D, S1E, and 1D, respectively), while p27^{Kip1} protein was drastically reduced in the pillar cell area of E18.5 Sox2:Dicer-cKO OC (Figure 1C, white arrow), where ongoing proliferation is occurring. Hence, impairment in miRNA biogenesis prevented p27^{Kip1} expression in the sensory progenitors and allowed a sustained proliferation within the sensory epithelium.

Cell differentiation in the cochlea begins at E14.5 and follows a base-to-apex gradient (Lim and Anniko, 1985). We performed immunolabelings to test whether Dicer-deficient progenitor cells within the OC are able to differentiate into HCs and SCs. Cochlear sections of E16.5 FoxG1:Dicer-cKO cochleae showed abnormal development with a significant increase in the number of HCs (Myosin6-positive) and obvious disorganization as compared to WT littermates (Figures 1D and 1E). Supernumerary HCs were also observed in Myo6-stained explants from Sox2:Dicer-cKO mice, confirming defective HC patterning in the absence of miRNAs (Figures 1G, white arrowhead, and 1H).

To decipher whether SC differentiation was impaired upon Dicer deletion, we performed immunolabelings to detect Prox1, p27^{Kip1}, or p75^{NTR} on cryosections from WT and FoxG1:Dicer-cKO E16.5 mouse embryos. The WT cochleae showed nuclear expression of both p27^{Kip1} and Prox1 in SCs (Figures 1D and S1F, respectively) (Bermingham-McDonogh et al., 2006), and p75^{NTR} was detected at the membrane of Hensen's cells and pillar cells (Figure S1F) (Mueller et al., 2002). In contrast, none of these proteins were immunodetected in E16.5 cochlear sections from FoxG1:Dicer-cKO animals (Figures 1D and S1F, respectively). Despite the lack of SCs marker expression, the prosensory markers Sox2 (Figure 1D) and Jagged1 (Jag1, Figure S1F) (Dabdoub et al., 2008) were detected in an enlarged domain upon Dicer deletion (Figures 1D, 1F, and S1F). Altogether, these results suggest that miRNAs are necessary for SC—but not for HC—differentiation.

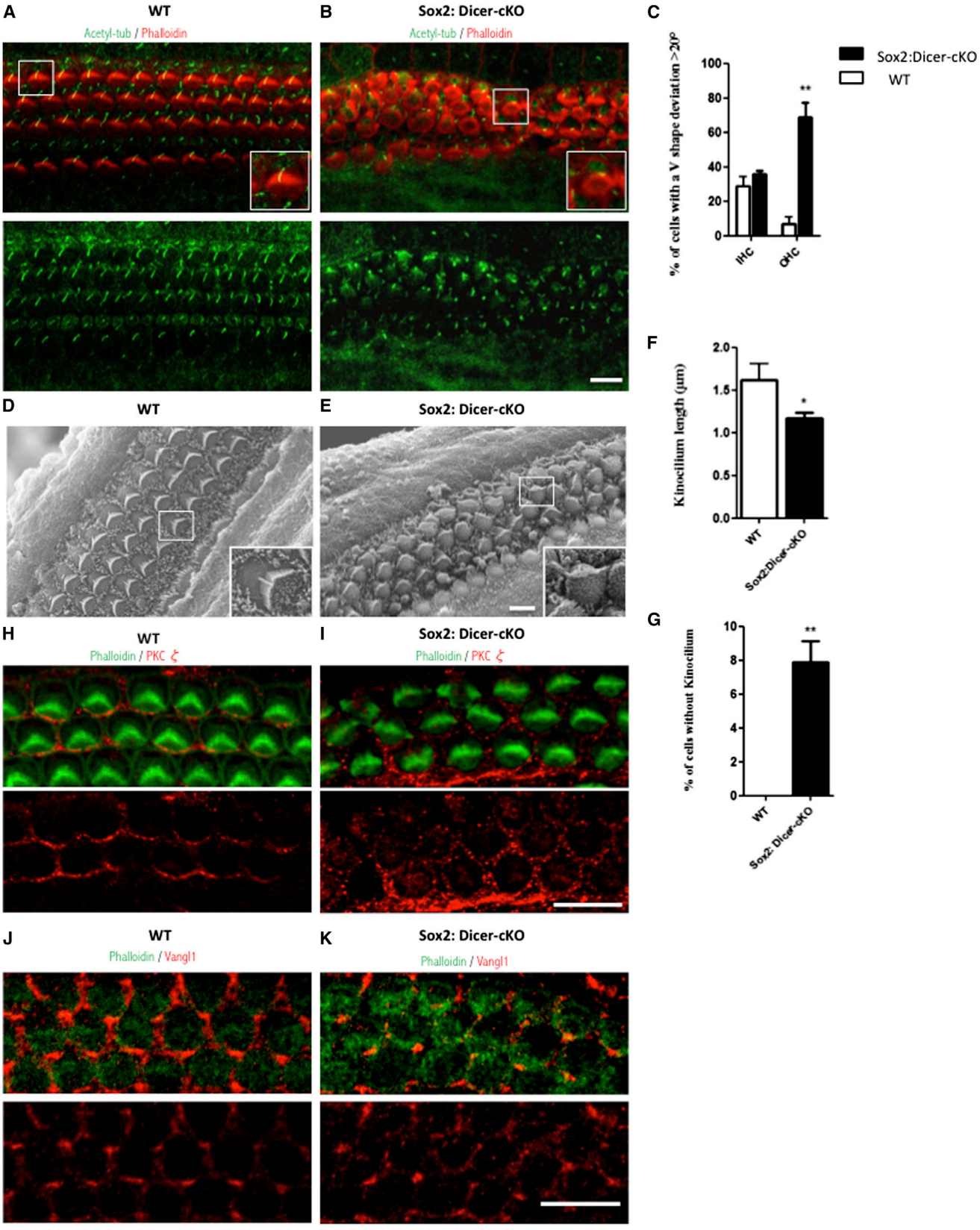
Loss of Dicer Results in Planar Cell Polarity-like Cochlear Defects

To further examine the role of Dicer in HC development, we sought to determine whether HC stereocilia bundles develop normally. At E18.5, the V-shaped hair bundles were observed in WT cochlea using phalloidin staining (Figure 2A). In Sox2:Dicer-cKO littermates, HCs displayed disoriented stereocilia bundles throughout the cochlear duct (Figures 2B and 2C). In addition, scanning electron microscopy (SEM) analysis revealed severe stereocilia bundle morphogenesis defects in the absence of Dicer (cf. Figures 2D and 2E). Indeed, some hair bundles appeared to be flattened (Figure 2E, insert) or disrupted (Figure 2I).

Figure 1. The Absence of miRNAs Induces Defects in Proliferation within the Sensory Epithelia and Affects the OC Differentiation

(A–C) Deletion of Dicer in the prosensory domain results in an increased cell proliferation. (A) Ki67/Sox2 double-labeled cochlear sections (left panels) or whole-mount preparations (right panels) from E18.5 Sox2:Dicer-cKO animals and WT control littermate. White arrows point proliferating cells between inner and outer hair cells (Myo6$^+$ or Parv$^+$) in Sox2:Dicer-cKO animals. BrdU incorporation (white arrowheads) confirms Ki67 labeling. (B) Quantification of proliferating cells in Sox2:Dicer-cKO and WT littermate demonstrated a significant increase of proliferation in the Sox2:Dicer-cKO OC ($p < 0.001$). (C) In the E18.5 Sox2:Dicer-cKO cochleae, the cell located in pillar cell region is negative for p27^{Kip1} labeling (white arrow).

(D–F) MicroRNAs promote appropriate differentiation of hair cells and supporting cells. (D) p27^{Kip1}-positive cells (green) are clearly absent from E16.5 FoxG1:Dicer-cKO cochleae, while supernumerary Myo6-positive (White) as well as Sox2-positive cells (red) are present. (E and F) The number of Myo6-positive cells (E) and Sox2-positive cells (F) is significantly increased in FoxG1:Dicer-cKO as compared to WT (*$p < 0.05$; **$p < 0.01$). (G and H) The number of HCs expressing Myo6 is also significantly increased in Sox2:Dicer-cKO (white arrowhead) as compared to WT littermates ($p < 0.05$). Data are presented as mean ± SEM; $n \geq 3$ in all genotypes; p values in relation to WT. Scale bars, 50 μm.



(legend on next page)

We also examined the position of the kinocilium and found that it was always associated with the center of the stereocilia bundle but that it could be located off the PCP axis in the cochlea in Sox2:Dicer-cKO mice (Figures 2A and 2B). Moreover, in many HCs, the kinocilium was either absent or stunted (Figures 2F and 2G). To confirm that PCP signaling is compromised in Dicer-cKO mice, we sought to examine the asymmetric membrane localization of the core PCP proteins Vangl1 and PKCζ. In E18.5 WT cochleae, these proteins were localized to the lateral side of HC membranes (Figures 2H and 2J). This localization was disrupted in Sox2:Dicer-cKO HCs (Figures 2I and 2K). Therefore, like core PCP genes, Dicer is important for coordinating the alignment of stereocilia bundles.

miR-124 Targets Sfrp4 and Sfpr5 Wnt Inhibitors

In order to identify which miRNA(s) and their target genes could be responsible of the Dicer-cKO cochlear phenotype, we performed two analytical approaches in parallel: (1) a microarray on total RNA extracts from E12.5 FoxG1:Dicer-cKO and WT cochlear littermates (Table S2) and (2) miRNA microarrays on WT OC at key developmental stages (i.e., E14.5, E16.5, and P1; Table S3).

Interestingly, we found that numerous components of the canonical and non-canonical Wnt pathways—known to be involved in cochlear development—were misexpressed in the absence of Dicer. Indeed, an increased expression of two inhibitors of Wnt signaling, *Sfrp4* and *Sfrp5*, was observed in FoxG1:Dicer-cKO cochleae as compared to WT littermates, whereas the expression of the Wnt ligands Wnt4, Wnt5a, Wnt7a, and Wnt7b and the Wnt co-receptors Lrp5 and Lrp6 was decreased (Figure 3A). In keeping with an overall inhibition of the Wnt pathway, the expression of the canonical Wnt target genes *Lgr5*, *Axin2*, *Eya1*, and *Fgf20*—known to be expressed in the inner ear (Chai et al., 2011; Chamorro et al., 2005; Freyer and Morrow, 2010)—was substantially decreased from −1.6- to −18-fold in the FoxG1:Dicer-cKO samples (Figure 3A). These results were confirmed by quantitative real-time PCR analysis (Figure S2). Altogether, these results suggest that the Wnt pathway may be fine-tuned by miRNAs during cochlear development.

To further identify which miRNAs target the Wnt pathway, focusing on its upregulated components upon deletion of Dicer (i.e., Sfrp4 and Sfrp5), involved in cochlear development, we analyzed miRNA microarrays by focusing our attention on highly expressed miRNAs or on miRNAs that present a pattern of expression compatible with a role in cochlear cell differentiation; i.e., high level at E14.5 or E16.5 and downregulation at P1 (Figure 3B; Table S3). Hence, we selected miR-200a, −200b, −200c, −335-5p, −182, and −124. In situ hybridizations confirmed that the expression level of miR-182 was very high in nascent HCs (Figure 3B) (Sacheli et al., 2009) and that all the other miRNAs analyzed were highly expressed in HCs (Figure 3B). On the contrary, none of these miRNAs were expressed in SCs. Taken together, these data further support a role for miRNAs in cochlear cell fate determination.

We then used target prediction programs (TargetScan and MiRanda) and luciferase assays to decipher which cochlear miRNAs of interest interact with Sfrp4/5. The miR-124 was predicted to target both Wnt inhibitors, whereas miR-200a and miR-141 were predicted to target *Sfrp4* (Figure 3C). Cotransfection of a reporter plasmid expressing the Renilla luciferase upstream of the 3′ UTR of Sfrp4 or Sfrp5 and plasmids expressing miR-124 specifically reduced luciferase activity in UB/OC1 cochlear cells (Figure 3D). Despite their bioinformatic prediction, miR-200a and miR-141 did not modify luciferase activity (Figure 3D). Moreover, mutations introduced into the putative miR-124 binding site of both the 3′ UTR of *Sfrp4* and *Sfrp5* (Figure 3C) abolished the repression (Figure 3D), indicating that Sfrp4 and 5 targeting by miR-124 is specific.

Next, we performed in situ hybridization for miR-124, *Sfrp4*, and *Sfrp5* in FoxG1:Dicer-cKO, Sox2:Dicer-cKO cochleae, and corresponding WT littermates, in order to analyze the biological relevance of this interaction. In WT cochleae, miR-124 was specifically present in nascent HCs, while *Sfrp4* and *Sfrp5* were absent (Figure 3E). Conversely, we detected an increased expression of *Sfrp4* and *Sfrp5* in both FoxG1: and Sox2:Dicer-cKO sensory epithelia, while miR-124 was not detected (Figure 3E). Altogether, our results suggest that miR-124 is expressed in the cochlear sensory epithelium, and particularly in HCs, where it represses *Sfrp4* and *Sfrp5* expression.

Wnt Inhibition Induces Abnormal Cell Specification and PCP Defects in the Cochlea

Having identified Sfrp4 and Sfrp5 as direct targets of miR-124, we decided to test whether Sfrp4 and Sfrp5 overexpression could recapitulate the phenotypic abnormalities observed in cochleae of Dicer cKO animals. For this purpose, cochlear explants were dissected before the time of HC differentiation, at E14.5, and cultured for 6 days in vitro (DIV) in the presence of recombinant Sfrp4 and Sfrp5 (rSfrp4/5) proteins. We first analyzed the effect of Sfrp4/5 on cell fate. We observed supernumerary Myo6-positive HCs in rSfrp4/5-treated explants as compared to untreated cultures (Figures 4A and 4B). These results were confirmed using DKK1, an antagonist of Wnt canonical signaling through interactions with LRP5/6 (Figures 4A and 4B). In addition, treatment with CHIR-99021, which activates the Wnt canonical pathway by selectively inhibiting GSK3β (Meijer et al.,

Figure 2. Planar Cell Polarity and Kinocilium Defects in Dicer Mutant Cochleae

(A–C) Surface view of E18.5 cochlear whole mount from WT (A) and Sox2:Dicer-cKO (B) mice stained with phalloidin (red) and acetylated alpha-tubulin (acetyl-tub) (green) to visualize the stereocilia and the kinocilium, respectively. The stereocilia bundles are misoriented and disorganized in the absence of Dicer. (C) Quantification of HC deviation from the polarity axis for WT and Sox2:Dicer-cKO animals ($p < 0.01$).

(D and E) Scanning electron microscopy of the basal turn of E18.5 cochleae from WT (D) and Sox2:Dicer-cKO mutant embryos (E). High-power views are shown to the right.

(F and G) Loss of Dicer caused a significant reduction of kinocilium length (F) as well as an increased number of hair cells without kinocilium (G).

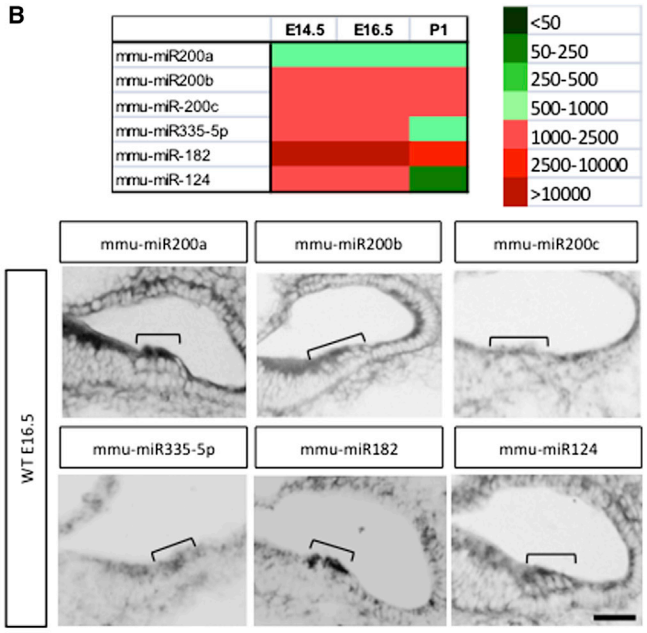
(H–K) Surface view of E18.5 cochleae from WT (H and J) and Sox2:Dicer-cKO (I, K) stained with phalloidin (green) and core PCP proteins (red) such as Vangl1 (cf. K to J) or PKC ζ (cf. I to H). Scale bars, 10 μm. Data are presented as mean ± SEM; $n \geq 3$ in all genotypes; p values in relation to WT (*$p < 0.05$; **$p < 0.01$).

A

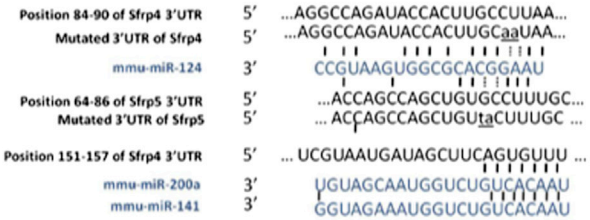
Fold change FoxG1:cKo Dicer vs WT cochlea

Inhibitors	Sfrp4	3
	Sfrp5	3,3
Ligands	Wnt4	-2,8
	Wnt5a	-1,7
	Wnt7a	-2,6
	Wnt7b	-3,7
Co-receptors	Lrp5	-1,6
	Lrp6	-1,6
Target genes	Lgr5	-2,5
	Axin2	-1,9
	Eya1	-2,8
	Fgf20	-18

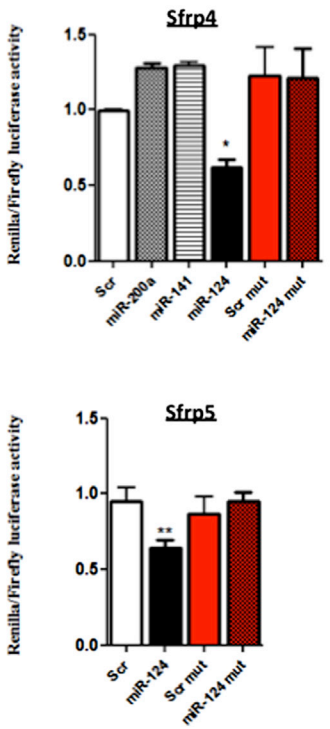
B



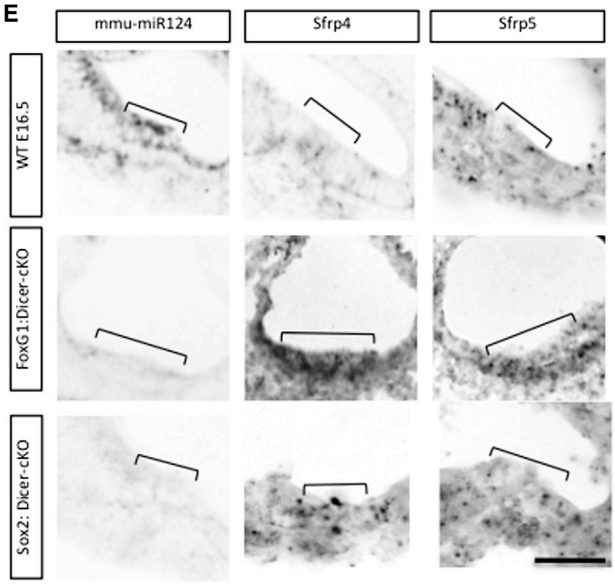
C



D



E



(legend on next page)

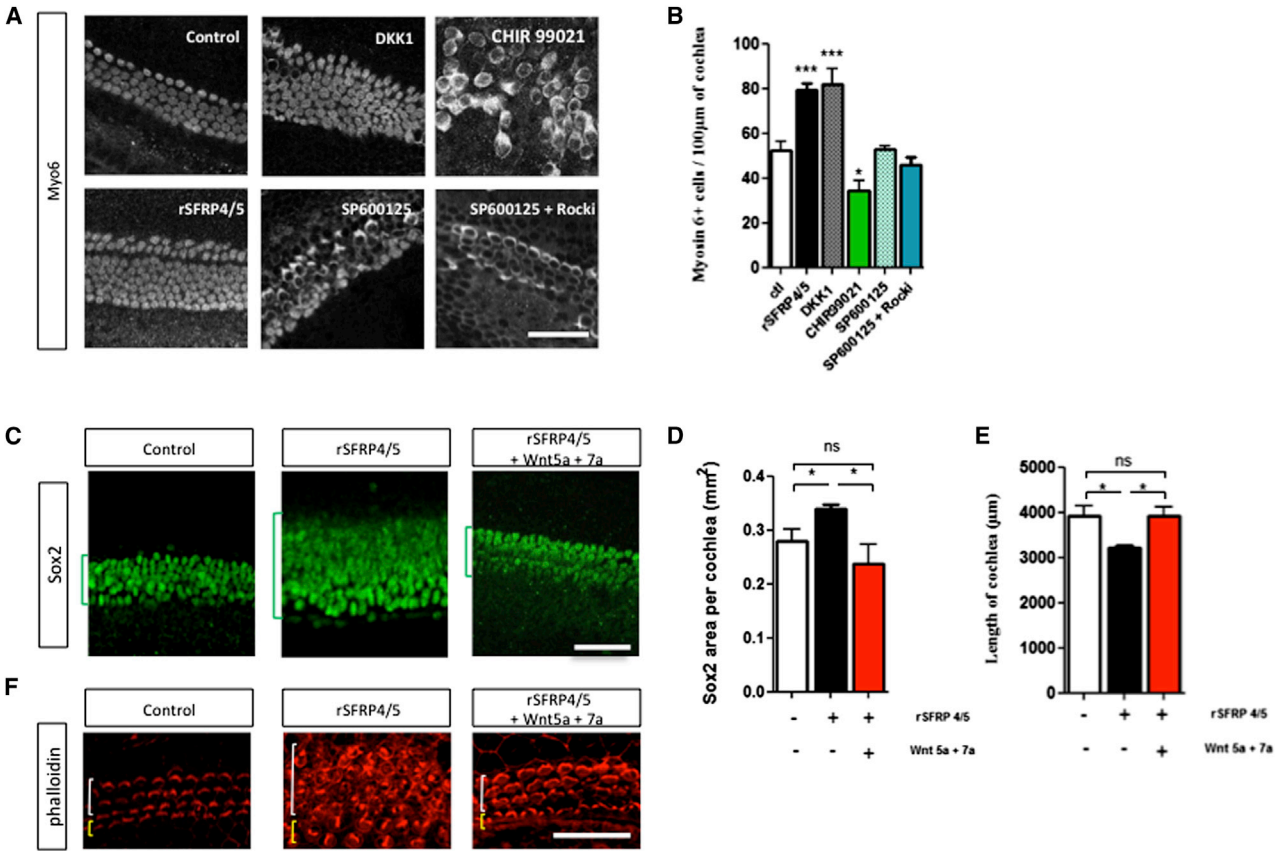


Figure 4. Effect of Wnt Signaling Inhibition on Cochlear Development

(A and B) E14.5 cochlear explants cultured for 6 days in the presence of recombinant proteins Sfrp4 and Sfrp5 (rSFRP4/5) (10 μg/ml each), Dkk1 (200 ng/ml), CHIR99021 (3 μm), or SP600125 and Rocki (10 μM each) and immunostained for Myo6 (A). The number of HCs ($MYO6^+$) are presented on the graphs (B) (*$p < 0.05$, ***$p < 0.001$).

(C–F) rSFRP4/5 treatment induces PCP defects in cultured organs of Corti that can be restored by Wnt5a and Wnt7a treatment. PCP defects comprise the enlargement of $Sox2^+$ sensory domain (green brackets in (C) and quantification in (D), $p < 0.05$); decreased cochlear length (E, $p < 0.05$) and the disruption of bundle orientation (F). Data are expressed as mean ± SEM. Scale bars, 50 μm.

2004), blocked HC differentiation. On the contrary, SP600125 (a JNK inhibitor) or Rocki, two specific inhibitors of the non-canonical Wnt pathway, had no effect on HC number. Altogether, these results indicate that Sfrp4/5 mimic the effect of Dicer knockdown on HC differentiation via an inhibition of the canonical Wnt pathway.

We then analyzed whether rSfrp4/5 could recapitulate the PCP defects observed in Dicer cKO cochleae. Interestingly, the sensory epithelia of Sfrp4/5-treated OC appeared wider (Figure 4C), with a significant increase of the Sox2 area per cochlea after 6 days of rSfrp4/5 treatment as compared to control condition (Figure 4D). In addition, exposure to rSfrp4/5 caused a shortening in the length of the cochlea (Figure 4E). The combined shortening in length and enlargement of width of the sensory epithelium suggests potential defects in the process of convergent extension, which is known to be regulated by the PCP pathway (Ezan and Montcouquiol, 2013). To determine whether these defects indeed correlate with reduced activity of

Figure 3. Sfrp4 and Sfrp5 Are Targets of miR-124 in the Developing Cochlea

(A) mRNA microarray results for Wnt signaling pathway on E12.5 WT and FoxG1:Dicer-cKO cochleae.

(B) Combination of microarray and in situ hybridizations identifying relevant cochlear miRNAs. Heatmaps showing the relative expression of miRNAs in the developing sensory epithelium between E14.5 and P1 (see also Table S3) and In situ hybridization performed on WT E16.5 transversal cochlear sections.

(C) Alignment of miR-124 and the 3′ UTR of *Sfrp4* or *Sfrp5 or* miR-200a/-141 and the 3′ UTR of *Sfrp4* showing putative binding sites. The mutations introduced in the 3′ UTR of *Sfrp4* and *Sfrp5* within the luciferase reporter vectors are indicated.

(D) Luciferase assays in UB/OC1 cells co-transfected with vectors coding for either the wild-type (WT) or mutated (MUT) 3′ UTR of *Sfrp4* (up) or *Sfrp5* (below) and corresponding miRNAs (as indicated). Data are expressed as mean ± SEM of at least three independent experiments. (*$p < 0.05$, **$p < 0.01$).

(E) In situ hybridizations for miR-124, *Sfrp4* and *Sfrp5* at different developmental stages in WT and Dicer-cKO cochlea. miR-124 is present in the HCs in WT mice while it is absent in FoxG1:Dicer-cKO at E16.5 and in Sox2:Dicer-cKO at E18.5. *Sfrp4* and *5*, weakly expressed in WT embryonic cochleae, are upregulated in the absence of Dicer. Brackets correspond to the sensory area of OC. Scale bars, 50 μm.

the Wnt/PCP pathway, we tested whether Wnt/PCP activation in response to Wnt-5a plus Wnt-7a (Wnt5a + 7a) could reverse rSfrp4/5 effects. As anticipated, rSfrp4/5 explants co-treated with Wnt-5a and -7a presented a normal cochlear length, and the orientation of the stereocilia bundle together with the number of Sox2-positive cells were both similar to untreated cultures (Figures 4C–4F). In addition, explants treated with both SP600125 and Rocki also present misoriented HC bundles (data not shown). Altogether, our findings show that rSfrp4/5 are able to mimic Dicer knockdown effects on cochlear development through a direct contribution of both the canonical and non-canonical Wnt pathways.

miR-124 Promotes Proper Cochlear Cell Differentiation by Targeting Sfrp4/5

To assess the role of miR-124 in cochlear cell specification and to test whether the loss of miR-124 expression could recapitulate the increased HC differentiation observed in the Dicer-cKO mutant mouse cochleae, we used "sponge" constructs designed to inhibit miR-124 (Spg-miR124) or a scramble miRNA (Spg-Scr) as previously described (Volvert et al., 2014). We first co-transfected the sponges in UB/OC1 cells along with miR-124 and a plasmid expressing the Renilla luciferase upstream of the 3′ UTR of *Sfrp4*. The reduced luciferase activity observed with miR-124 was significantly reverted in the presence of Spg-miR124 (Figure S3). We then electroporated E14.5 WT cochleae with Spg-miR124 or Spg-Scr and cultured them for 6DIV. Electroporation of Spg-miR124 resulted in the expression of Myo6 in 39.2% ± 3.8% of the transfected cells, while explants electroporated with the Spg-Scr only presented 24.4% ± 2.8% of Myo6-positive cells, indicating that a reduction in miR124 availability among cochlear prosensory cells induced a switch toward the HC fate (Figures 5A and 5B). Importantly, concurrent neutralization of endogenous miR-124 and inhibition of Sfrp4/5 expression using specific short hairpin RNA (shRNA) vectors rescued normal HC differentiation (Figures 5C and 5D). Altogether, these results suggest that a tight regulation of *Sfrp4/5* expression by miR-124 is required for appropriate HC specification within the cochlea and that the increased HC differentiation observed in the absence of Dicer could be attributed to the lack of miR-124 maturation (Figure 5E).

DISCUSSION

In this study, we describe a new function of the microRNA machinery in the development of the OC. Conditional removal of *Dicer* combined with global gene expression profiling and miRNA expression pattern analysis allowed us to identify miR-124 as a critical regulator of cochlear development. This miRNA controls cochlear cell patterning, PCP and ciliogenesis in the cochlea by specifically repressing Sfrp4 and Sfrp5 inhibitors of the Wnt pathway.

MicroRNAs Act as Key Regulators of Proliferation and Differentiation in the Developing OC

When prosensory cells were deprived of miRNAs after their cell-cycle exit, both in FoxG1: and Sox2:Dicer-cKO mouse models, an excessive proliferation was found. This correlates with a reduced or missing $p27^{Kip1}$ expression that is sufficient to explain the sustained proliferation observed in Dicer-cKO cochleae as previously reported in $p27^{-/-}$ mice (Löwenheim et al., 1999).

The supernumerary progenitors, arising from this delayed cell proliferation, retain the capacity to differentiate into HCs. Intriguingly, the cells surrounding the HCs within the OC of FoxG1:Dicer-cKO cochleae do not express any of the known specific markers for SCs. The expression of Sox2 and Jag1 proteins rather suggest that these cells remain in a progenitor state. This exclusive role for Dicer in SC development cannot be attributed to the lack of $p27^{Kip1}$ protein since Prox1-positive cells, a specific SC marker (Bermingham-McDonogh et al., 2006), are present in $p27^{-/-}$ mice (unpublished data).

MicroRNAs Regulate PCP and Kinocilium Formation in the Cochlea

Our findings indicate that the integrity of the miRNA machinery is also essential to preserve PCP signaling in the cochlea. These defects in convergent extension and HCs misorientation are similar to those observed in mice carrying mutant alleles of core PCP genes such as Vangl2 or Celsr1 (Curtin et al., 2003; Montcouquiol et al., 2003). Kinocilium development may also be regulated by microRNAs since we observed that targeted disruption of Dicer in the OC shortened kinocilium length. Whether Dicer is localized within the kinocilium remains to be determined. However, previous studies have shown that some proteins of the miRNA machinery are present in the primary cilium (Moser et al., 2011). In addition, several recent studies showed that cell-type-specific miRNA deficiency results in defective ciliogenesis (Walentek et al., 2014). It remains to decipher whether miRNAs act directly on both PCP and ciliogenesis since these pathways may be functionally related. Indeed, PCP effector proteins have been demonstrated to have a role on ciliogenesis (Heydeck et al., 2009; Tissir et al., 2010), and defective kinocilium genesis impairs HC orientation within the OC (Jones et al., 2008). However, in the absence of important ciliary proteins, the asymmetric localization of core PCP proteins is maintained (Borovina and Ciruna, 2013).

miR-124 Fine-Tunes Canonical and Non-canonical Wnt Pathways

Wnt signaling inhibitors Sfrp4/5 were the only genes of well-known regulators of the development of the organ of Corti whose expression was upregulated in Dicer-cKO cochleae. This is correlated with a significant reduction of Wnt ligands and well-known Wnt target genes, demonstrating that the Wnt pathway is inhibited in the absence of miRNAs. In parallel, we showed that Sfrp4/5 are specifically targeted by miR-124. It is noteworthy that miR-124 is highly expressed in future HCs (i.e., at E16.5), while Sfrp4/5 are excluded in WT cochleae. On the contrary, in Dicer-cKO mice, where miR-124 is absent, or after blocking endogenous miR-124 in cultured explants, Sfrp4/5 are derepressed and this leads to the production of supernumerary HCs. This is consistent with other studies showing that miR-124 promotes neuronal differentiation in the nervous system (Cheng et al., 2009; Makeyev et al., 2007). Surprisingly, while miR-124 appears to directly target Sox9 and Jag1 in the CNS, we did not detect any variation of those two specific markers

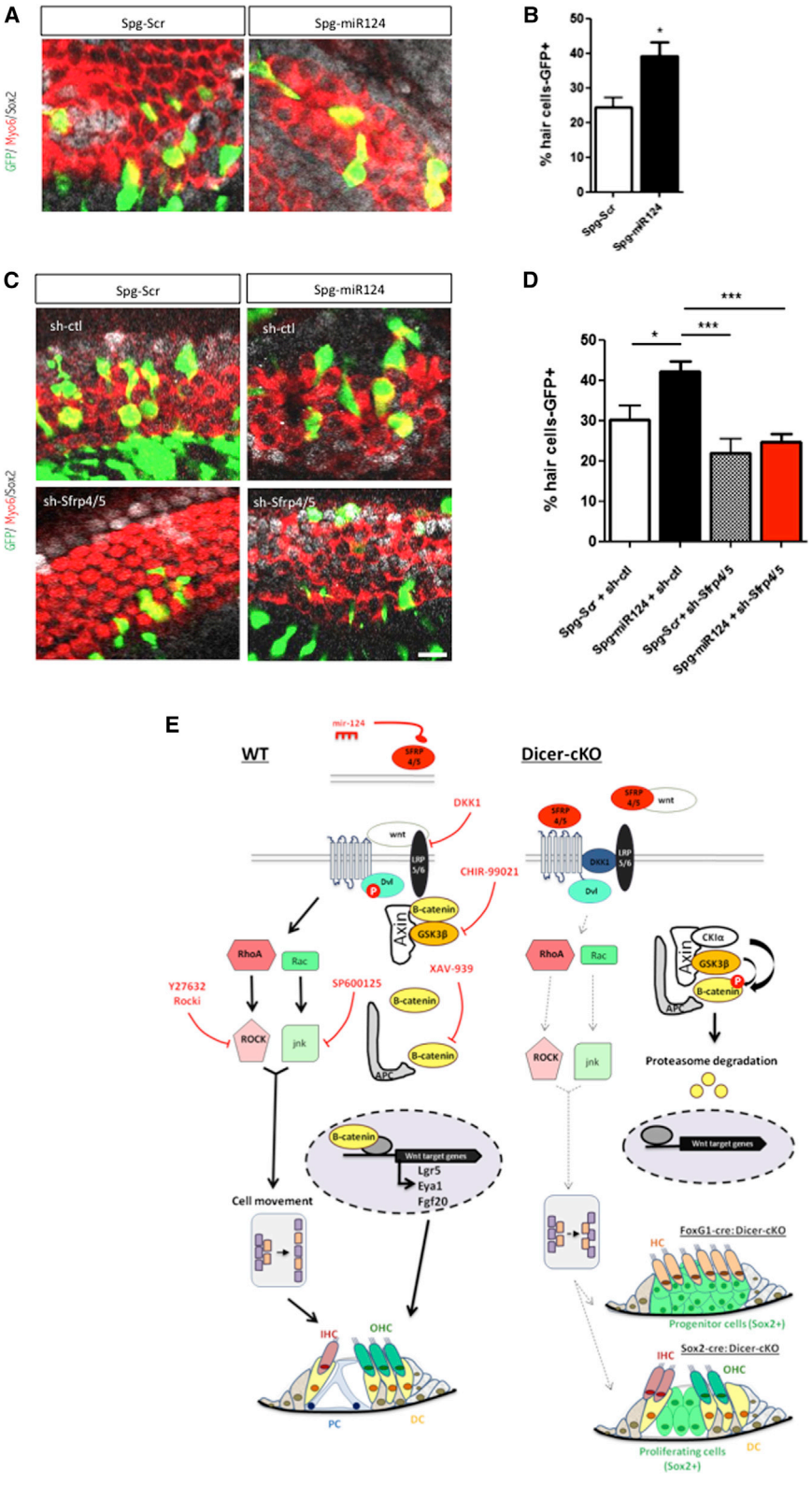


Figure 5. Hair Cell Fate Is Promoted by miR-124 Inhibition

(A) E14.5 explants electroporated with sponges miR-Scr (Spg-Scr) or miR-124 (Spg-miR124) constructs carrying a GFP transgene and further cultured for 6 days to allow cellular differentiation.

(B) The percentage of transfected cells (GFP^+) that differentiate into HCs ($Myo6^+$) is increased in Spg-miR124 electroporated explants as compared to Spg-Scr ($p < 0.05$).

(C) The regulation of *Sfrp4/5* expression by miR-124 is required for appropriate cell fate specification within the cochlea. E14.5 cochleae were co-electroporated with Spg-Scr or Spg-miR124 and shRNA-control (sh-ctl) or SFRP4/5-ShRNA (sh-Sfrp4/5) targeting vectors.

(D) Histograms presenting the percentage of GFP-positive cells that differentiate into HCs (*$p < 0.05$; ***$p < 0.001$). Data are expressed as mean ± SEM. Scale bars, 10 μm.

(E) Summary diagram illustrating how Dicer and miR-124 control cell patterning through Wnt signaling pathway in the developing organ of Corti.

of progenitors and SCs (Mak et al., 2009; Morrison et al., 1999)—both by microarrays and immunostainings—in the OC of Dicer-cKO mice. This means that the regulation of target genes by miR-124 may be tissue specific or that its effect is counteracted by another tissue-specific miRNA.

Among the direct canonical Wnt/β-catenin target genes, we found that Fgf20 and Lgr5 are downregulated in Dicer-cKO cochleae. Interestingly, blocking Fgf20 signaling inhibits SC development in cultured OC (Hayashi et al., 2008), while a specific increase in IHC number is observed in Fgf20 KO mice (Huh et al., 2012), similarly to the one we observed in Sox2:Dicer-cKO mice. Moreover, Lgr5 is specifically expressed in SCs and Lgr5-expressing cells can generate new HCs via transdifferentiation (Shi et al., 2012). It is important to mention that the Wnt signaling pathway has been largely involved in cochlear cell differentiation (Jansson et al., 2015). Surprisingly, manipulation of this signaling pathway gives rise to opposite results in a time-dependent manner. Indeed, upregulating Wnt signaling at E12.5 caused increased HC differentiation (Jacques et al., 2012; Shi et al., 2014), whereas its manipulation at E13.5 (Mulvaney et al., 2013) or E14.5 (current work) impairs HC differentiation. How can results be reconciled with contradictory effects? Reasonable assumptions are that (1) Wnt signaling pathway oscillates during cochlear development as previously demonstrated in other tissues (Aulicino et al., 2014) or that (2) a shift away from canonical Wnt signaling toward noncanonical pathways occurs at E13.5–E14.5 (Qian et al., 2007), leading to an opposite effect on HC differentiation.

The PCP and kinocilium defects observed in Dicer-cKO mice can also be explained by the upregulation of Sfrp4/5. Indeed, the Wnt/PCP non-canonical pathway plays a major role during the development of the cochlea. It has been demonstrated that Wnt5a KO mice present a shortened cochlear duct and misaligned stereocilia bundles, while in vitro treatment of E13 cochleae with Wnt signaling inhibitors such as Sfrp1 or Wif1 disrupts stereocilia bundle orientation (Dabdoub et al., 2003). Moreover, E14.5 Sfrp3-treated cochleae display abnormal polarization of stereocilia associated with a decrease of the cochlear length (Qian et al., 2007), similarly to our results obtained with Sfrp4/5-treated cochleae and Dicer-cKO cochleae.

Although we cannot exclude the fine-tuning of additional targets by other miRNAs during late embryonic cochlear development, the present findings indicate that miR-124 plays a critical role in late cochlear development by modulating the expression of Sfrp4/5 and thereby affecting Wnt signaling pathways. In conclusion, our work emphasizes the crucial roles of miRNAs in regulating the mechanisms that govern cell patterning in the mammalian OC. These findings may provide us with clues to help the development of new therapeutic strategies but also point out new avenues for HC reprogramming aimed at restoring hearing.

EXPERIMENTAL PROCEDURES

More detailed methods are described in the Supplemental Experimental Procedures.

Mouse Lines

Timed-pregnant NMRI (Janvier labs), ROSAR26R-yellow fluorescent protein (YFP; R26R-YFP) (S. Srivinas), Dicerflox/flox (M. Merkenschlager), Foxg1Cre/+ (J.-M. Hébert), and Sox2CreERT2/+ mice (S.K. Nikolis) were housed under standard conditions and bred in accordance with the declaration of Helsinki and following the guidelines of the Belgian ministry of agriculture in agreement with EC laboratory animal care and use regulation (86/609/CEE, CE of J no. L358, December 18, 1986).

Statistics

All data are reported as mean ± SEM. Statistical analyses were performed using GraphPad InStat software (GraphPad). Student's t tests were used for paired comparisons and one-way ANOVA followed by a Tukey's post hoc test was used for multiple comparisons.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, three figures, and three tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2015.08.054.

AUTHOR CONTRIBUTIONS

A.H., P.V.d.A., R.S., P.P.P., L.D., L.N., and B.M. conceived and designed the experiments. A.H., P.V.d.A., R.S., P.P.P., L.D., J.R., N.T., and M.T. performed the experiments. A.H., P.V.d.A., R.S., L.D., and B.M. analyzed the data. A.H., P.V.d.A., L.D., L.N., and B.M. wrote the paper.

ACKNOWLEDGMENTS

The authors thank P.B. Van Lerberghe for technical assistance and S. Freeman for critical reading of the manuscript. We thank J.M. Hébert, S. Srivinas, M. Merkenschlager, and S.K. Nikolis for sharing cre-mouse lines and L. Leyns for providing plasmids. We thank the GIGA-Imaging and Flow Cytometry platform for technical support. L.N. and B.M. are Research Associate and Senior Research Associate from the Belgian National Funds for Scientific Research (FRS-FNRS), respectively. B.M. is funded by grants from the FRS-FNRS, the Fonds Léon Fredericq, the Fondation Médicale Reine Elisabeth, and the Belgian Science Policy (IAP-VII network P7/07). L.N. is funded by a grant from ARC (ARC11/16-01).

Received: May 29, 2015
Revised: July 1, 2015
Accepted: August 18, 2015
Published: September 17, 2015

REFERENCES

Arnold, K., Sarkar, A., Yram, M.A., Polo, J.M., Bronson, R., Sengupta, S., Seandel, M., Geijsen, N., and Hochedlinger, K. (2011). Sox2(+) adult stem and progenitor cells are important for tissue regeneration and survival of mice. Cell Stem Cell *9*, 317–329.

Aulicino, F., Theka, I., Ombrato, L., Lluis, F., and Cosma, M.P. (2014). Temporal perturbation of the Wnt signaling pathway in the control of cell reprogramming is modulated by TCF1. Stem Cell Reports *2*, 707–720.

Bartel, D.P. (2004). MicroRNAs: genomics, biogenesis, mechanism, and function. Cell *116*, 281–297.

Bermingham, N.A., Hassan, B.A., Price, S.D., Vollrath, M.A., Ben-Arie, N., Eatock, R.A., Bellen, H.J., Lysakowski, A., and Zoghbi, H.Y. (1999). Math1: an essential gene for the generation of inner ear hair cells. Science *284*, 1837–1841.

Bermingham-McDonogh, O., Oesterle, E.C., Stone, J.S., Hume, C.R., Huynh, H.M., and Hayashi, T. (2006). Expression of Prox1 during mouse cochlear development. J. Comp. Neurol. *496*, 172–186.

Borovina, A., and Ciruna, B. (2013). IFT88 plays a cilia- and PCP-independent role in controlling oriented cell divisions during vertebrate embryonic development. Cell Rep. *5*, 37–43.

Chai, R., Xia, A., Wang, T., Jan, T.A., Hayashi, T., Bermingham-McDonogh, O., and Cheng, A.G. (2011). Dynamic expression of Lgr5, a Wnt target gene, in the developing and mature mouse cochlea. J. Assoc. Res. Otolaryngol. *12*, 455–469.

Chamorro, M.N., Schwartz, D.R., Vonica, A., Brivanlou, A.H., Cho, K.R., and Varmus, H.E. (2005). FGF-20 and DKK1 are transcriptional targets of beta-catenin and FGF-20 is implicated in cancer and development. EMBO J. *24*, 73–84.

Cheng, L.C., Pastrana, E., Tavazoie, M., and Doetsch, F. (2009). miR-124 regulates adult neurogenesis in the subventricular zone stem cell niche. Nat. Neurosci. *12*, 399–408.

Cobb, B.S., Nesterova, T.B., Thompson, E., Hertweck, A., O'Connor, E., Godwin, J., Wilson, C.B., Brockdorff, N., Fisher, A.G., Smale, S.T., and Merkenschlager, M. (2005). T cell lineage choice and differentiation in the absence of the RNase III enzyme Dicer. J. Exp. Med. *201*, 1367–1373.

Curtin, J.A., Quint, E., Tsipouri, V., Arkell, R.M., Cattanach, B., Copp, A.J., Henderson, D.J., Spurr, N., Stanier, P., Fisher, E.M., et al. (2003). Mutation of Celsr1 disrupts planar polarity of inner ear hair cells and causes severe neural tube defects in the mouse. Curr. Biol. *13*, 1129–1133.

Dabdoub, A., Donohue, M.J., Brennan, A., Wolf, V., Montcouquiol, M., Sassoon, D.A., Hseih, J.C., Rubin, J.S., Salinas, P.C., and Kelley, M.W. (2003). Wnt signaling mediates reorientation of outer hair cell stereociliary bundles in the mammalian cochlea. Development *130*, 2375–2384.

Dabdoub, A., Puligilla, C., Jones, J.M., Fritzsch, B., Cheah, K.S., Pevny, L.H., and Kelley, M.W. (2008). Sox2 signaling in prosensory domain specification and subsequent hair cell differentiation in the developing cochlea. Proc. Natl. Acad. Sci. USA *105*, 18396–18401.

Ezan, J., and Montcouquiol, M. (2013). Revisiting planar cell polarity in the inner ear. Semin. Cell Dev. Biol. *24*, 499–506.

Freyer, L., and Morrow, B.E. (2010). Canonical Wnt signaling modulates Tbx1, Eya1, and Six1 expression, restricting neurogenesis in the otic vesicle. Dev. Dyn. *239*, 1708–1722.

Friedman, L.M., Dror, A.A., Mor, E., Tenne, T., Toren, G., Satoh, T., Biesemeier, D.J., Shomron, N., Fekete, D.M., Hornstein, E., and Avraham, K.B. (2009). MicroRNAs are essential for development and function of inner ear hair cells in vertebrates. Proc. Natl. Acad. Sci. USA *106*, 7915–7920.

Groves, A.K., Zhang, K.D., and Fekete, D.M. (2013). The genetics of hair cell development and regeneration. Annu. Rev. Neurosci. *36*, 361–381.

Hayashi, T., Ray, C.A., and Bermingham-McDonogh, O. (2008). Fgf20 is required for sensory epithelial specification in the developing cochlea. J. Neurosci. *28*, 5991–5999.

Hébert, J.M., and McConnell, S.K. (2000). Targeting of cre to the Foxg1 (BF-1) locus mediates loxP recombination in the telencephalon and other developing head structures. Dev. Biol. *222*, 296–306.

Heydeck, W., Zeng, H., and Liu, A. (2009). Planar cell polarity effector gene Fuzzy regulates cilia formation and Hedgehog signal transduction in mouse. Dev. Dyn. *238*, 3035–3042.

Huh, S.H., Jones, J., Warchol, M.E., and Ornitz, D.M. (2012). Differentiation of the lateral compartment of the cochlea requires a temporally restricted FGF20 signal. PLoS Biol. *10*, e1001231.

Jacques, B.E., Puligilla, C., Weichert, R.M., Ferrer-Vaquer, A., Hadjantonakis, A.K., Kelley, M.W., and Dabdoub, A. (2012). A dual function for canonical Wnt/β-catenin signaling in the developing mammalian cochlea. Development *139*, 4395–4404.

Jansson, L., Kim, G.S., and Cheng, A.G. (2015). Making sense of Wnt signaling-linking hair cell regeneration to development. Front. Cell. Neurosci. *9*, 66.

Jones, C., Roper, V.C., Foucher, I., Qian, D., Banizs, B., Petit, C., Yoder, B.K., and Chen, P. (2008). Ciliary proteins link basal body polarization to planar cell polarity regulation. Nat. Genet. *40*, 69–77.

Kersigo, J., D'Angelo, A., Gray, B.D., Soukup, G.A., and Fritzsch, B. (2011). The role of sensory organs and the forebrain for the development of the craniofacial shape as revealed by Foxg1-cre-mediated microRNA loss. Genesis *49*, 326–341.

Kuhn, S., Johnson, S.L., Furness, D.N., Chen, J., Ingham, N., Hilton, J.M., Steffes, G., Lewis, M.A., Zampini, V., Hackney, C.M., et al. (2011). miR-96 regulates the progression of differentiation in mammalian cochlear inner and outer hair cells. Proc. Natl. Acad. Sci. USA *108*, 2355–2360.

Lewis, M.A., Quint, E., Glazier, A.M., Fuchs, H., De Angelis, M.H., Langford, C., van Dongen, S., Abreu-Goodger, C., Piipari, M., Redshaw, N., et al. (2009). An ENU-induced mutation of miR-96 associated with progressive hearing loss in mice. Nat. Genet. *41*, 614–618.

Li, H., and Fekete, D.M. (2010). MicroRNAs in hair cell development and deafness. Curr. Opin. Otolaryngol. Head Neck Surg. *18*, 459–465.

Lim, D.J., and Anniko, M. (1985). Developmental morphology of the mouse inner ear. A scanning electron microscopic observation. Acta Otolaryngol. Suppl. *422*, 1–69.

Löwenheim, H., Furness, D.N., Kil, J., Zinn, C., Gültig, K., Fero, M.L., Frost, D., Gummer, A.W., Roberts, J.M., Rubel, E.W., et al. (1999). Gene disruption of p27(Kip1) allows cell proliferation in the postnatal and adult organ of corti. Proc. Natl. Acad. Sci. USA *96*, 4084–4088.

Mak, A.C., Szeto, I.Y., Fritzsch, B., and Cheah, K.S. (2009). Differential and overlapping expression pattern of SOX2 and SOX9 in inner ear development. Gene Expr. Patterns *9*, 444–453.

Makeyev, E.V., Zhang, J., Carrasco, M.A., and Maniatis, T. (2007). The MicroRNA miR-124 promotes neuronal differentiation by triggering brain-specific alternative pre-mRNA splicing. Mol. Cell *27*, 435–448.

Meijer, L., Flajolet, M., and Greengard, P. (2004). Pharmacological inhibitors of glycogen synthase kinase 3. Trends Pharmacol. Sci. *25*, 471–480.

Mencía, A., Modamio-Høybjør, S., Redshaw, N., Morín, M., Mayo-Merino, F., Olavarrieta, L., Aguirre, L.A., del Castillo, I., Steel, K.P., Dalmay, T., et al. (2009). Mutations in the seed region of human miR-96 are responsible for nonsyndromic progressive hearing loss. Nat. Genet. *41*, 609–613.

Montcouquiol, M., Rachel, R.A., Lanford, P.J., Copeland, N.G., Jenkins, N.A., and Kelley, M.W. (2003). Identification of Vangl2 and Scrb1 as planar polarity genes in mammals. Nature *423*, 173–177.

Morrison, A., Hodgetts, C., Gossler, A., Hrabé de Angelis, M., and Lewis, J. (1999). Expression of Delta1 and Serrate1 (Jagged1) in the mouse inner ear. Mech. Dev. *84*, 169–172.

Moser, J.J., Fritzler, M.J., and Rattner, J.B. (2011). Repression of GW/P body components and the RNAi microprocessor impacts primary ciliogenesis in human astrocytes. BMC Cell Biol. *12*, 37.

Mueller, K.L., Jacques, B.E., and Kelley, M.W. (2002). Fibroblast growth factor signaling regulates pillar cell development in the organ of corti. J. Neurosci. *22*, 9368–9377.

Mulvaney, J.F., Yatteau, A., Sun, W.W., Jacques, B., Takubo, K., Suda, T., Yamada, W., and Dabdoub, A. (2013). Secreted factor R-Spondin 2 is involved in refinement of patterning of the mammalian cochlea. Dev. Dyn. *242*, 179–188.

Qian, D., Jones, C., Rzadzinska, A., Mark, S., Zhang, X., Steel, K.P., Dai, X., and Chen, P. (2007). Wnt5a functions in planar cell polarity regulation in mice. Dev. Biol. *306*, 121–133.

Ruben, R.J. (1967). Development of the inner ear of the mouse: a radioautographic study of terminal mitoses. Acta Otolaryngol. *220*, 220, 1–44.

Sacheli, R., Nguyen, L., Borgs, L., Vandenbosch, R., Bodson, M., Lefebvre, P., and Malgrange, B. (2009). Expression patterns of miR-96, miR-182 and miR-183 in the development inner ear. Gene Expr. Patterns *9*, 364–370.

Shi, F., Kempfle, J.S., and Edge, A.S. (2012). Wnt-responsive Lgr5-expressing stem cells are hair cell progenitors in the cochlea. J. Neurosci. *32*, 9639–9648.

Shi, F., Hu, L., Jacques, B.E., Mulvaney, J.F., Dabdoub, A., and Edge, A.S. (2014). β-Catenin is required for hair-cell differentiation in the cochlea. J. Neurosci. *34*, 6470–6479.

Sinkkonen, S.T., Chai, R., Jan, T.A., Hartman, B.H., Laske, R.D., Gahlen, F., Sinkkonen, W., Cheng, A.G., Oshima, K., and Heller, S. (2011). Intrinsic regenerative potential of murine cochlear supporting cells. Sci. Rep. *1*, 26.

Soukup, G.A., Fritzsch, B., Pierce, M.L., Weston, M.D., Jahan, I., McManus, M.T., and Harfe, B.D. (2009). Residual microRNA expression dictates the

extent of inner ear development in conditional Dicer knockout mice. Dev. Biol. *328*, 328–341.

Tissir, F., Qu, Y., Montcouquiol, M., Zhou, L., Komatsu, K., Shi, D., Fujimori, T., Labeau, J., Tyteca, D., Courtoy, P., et al. (2010). Lack of cadherins Celsr2 and Celsr3 impairs ependymal ciliogenesis, leading to fatal hydrocephalus. Nat. Neurosci. *13*, 700–707.

Volvert, M.L., Prévot, P.P., Close, P., Laguesse, S., Pirotte, S., Hemphill, J., Rogister, F., Kruzy, N., Sacheli, R., Moonen, G., et al. (2014). MicroRNA targeting of CoREST controls polarization of migrating cortical neurons. Cell Rep. *7*, 1168–1183.

Walentek, P., Song, R., and He, L. (2014). microRNAs and cilia. An ancient connection. Cell Cycle *13*, 2315–2316.

Wallingford, J.B. (2012). Planar cell polarity and the developmental control of cell behavior in vertebrate embryos. Annu. Rev. Cell Dev. Biol. *28*, 627–653.

Weston, M.D., Pierce, M.L., Rocha-Sanchez, S., Beisel, K.W., and Soukup, G.A. (2006). MicroRNA gene expression in the mouse inner ear. Brain Res. *1111*, 95–104.

Weston, M.D., Pierce, M.L., Jensen-Smith, H.C., Fritzsch, B., Rocha-Sanchez, S., Beisel, K.W., and Soukup, G.A. (2011). MicroRNA-183 family expression in hair cell development and requirement of microRNAs for hair cell maintenance and survival. Dev. Dyn. *240*, 808–819.

Zine, A., Van De Water, T.R., and de Ribaupierre, F. (2000). Notch signaling regulates the pattern of auditory hair cell differentiation in mammals. Development *127*, 3373–3383.