

1 **Dual expression of Atoh1 and Ikzf2 promotes transformation of adult**  
2 **cochlear supporting cells into outer hair cells**

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28 **ABSTRACT**

29 Mammalian cochlear outer hair cells (OHCs) are essential for hearing. OHC  
30 degeneration causes severe hearing impairment. Previous attempts of regenerating new  
31 OHCs from cochlear supporting cells (SCs) had yielded cells lacking Prestin, a key  
32 motor protein for OHC function. Thus, regeneration of Prestin+ OHCs remains a  
33 challenge for repairing OHC damage *in vivo*. Here, we reported that successful *in vivo*  
34 conversion of adult cochlear SCs into Prestin+ OHC-like cells could be achieved by  
35 simultaneous expression of Atoh1 and Ikzf2, two key transcriptional factors necessary  
36 for OHC development. New OHC-like cells exhibited upregulation of hundreds of  
37 OHC genes and downregulation of SC genes. Single cell transcriptomic analysis  
38 demonstrated that the differentiation status of these OHC-like cells was much more  
39 advanced than previously achieved. Thus, we have established an efficient approach to  
40 promote regeneration of Prestin+ OHCs and paved the way for repairing damaged  
41 cochlea *in vivo* via transdifferentiation of SCs.

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## 50 INTRODUCTION

51 Hair cells (HCs) are mammalian sound receptors that are distributed in the auditory  
52 epithelium, referred to as the organ of Corti (OC) (Wu and Kelley, 2012). Located near  
53 HCs are several supporting cell (SC) subtypes, which, from the medial side to lateral  
54 side, are Pillar cells (PCs) and Deiters' cells (DCs) (Montcouquiol and Kelley, 2020).  
55 Auditory HCs comprise two subtypes, the inner and outer HCs (IHCs and OHCs).  
56 OHCs specifically express a unique motor protein, Prestin, encoded by *Slc26a5* (Zheng  
57 et al., 2000). Prestin-mediated electromotility enables OHCs to function as sound  
58 amplifiers, which is critical for sound detection, and *Prestin*<sup>-/-</sup> mice show severe hearing  
59 impairment (Liberman et al., 2002). Different from OHCs, IHCs are primary sensory  
60 cells and are innervated by type-I cochlear spiral ganglion neurons (SGNs). IHCs  
61 specifically express vGlut3, encoded by *Slc17a8*, which is required for sound  
62 information transition from IHCs to SGNs (Li et al., 2018); consequently, *vGlut3*<sup>-/-</sup> mice  
63 are completely deaf (Ruel et al., 2008; Seal et al., 2008). IHCs and OHCs are considered  
64 to share the same Atoh1+ progenitors (Groves et al., 2013; Tateya et al., 2019).

65 Atoh1 is a bHLH transcriptional factor (TF) that is necessary for specifying a  
66 general HC fate, and, accordingly, both IHCs and OHCs are lost in *Atoh1*<sup>-/-</sup> mice  
67 (Bermingham et al., 1999). Two additional TFs, encoded by *Insm1* and *Ikzf2*, are  
68 necessary for specifying the OHC fate or repressing the IHC fate (Chessum et al., 2018;  
69 Wiwatpanit et al., 2018). OHCs tend to transdifferentiate into IHCs in *Insm1*<sup>-/-</sup> and *Ikzf2*  
70 point-mutant mice. Whereas *Insm1* is only transiently expressed in differentiating  
71 OHCs (Lorenzen et al., 2015), *Ikzf2* expression is permanently maintained in

72 differentiating and mature OHCs (Chessim et al., 2018). Unlike IHCs, OHCs are highly  
73 vulnerable to ototoxic drugs, noise, and aging. Nonmammal vertebrates such as fish  
74 and chicken can regenerate HCs from neighboring SCs in which key HC developmental  
75 genes (e.g. *Atoh1*) are reactivated (Atkinson et al., 2015), whereas mammals have lost  
76 this regenerative capacity (Janesick and Heller, 2019). PCs and DCs are physically close  
77 to OHCs, and, notably, OHCs, PCs, and DCs might share the same progenitors located  
78 in the lateral side of the OC, according to the results of recent single-cell transcriptomic  
79 analyses of cochlear cells (Kolla et al., 2020). Therefore, PCs and DCs, particularly  
80 these *Lgr5*<sup>+</sup> populations, are regarded as a favorable source for regenerating OHCs  
81 (Chai et al., 2012; McLean et al., 2017). We have shown that *in vivo* ectopic *Atoh1* can  
82 convert neonatal and juvenile SCs (primarily PCs and DCs) into nascent HCs that  
83 express early HC markers such as Myo6 and Myo7a (Liu et al., 2012a). By contrast,  
84 adult PCs and DCs are not sensitive to ectopic *Atoh1* expression *in vivo* (Kelly et al.,  
85 2012; Liu et al., 2012a), unless additional manipulations are performed (Walters et al.,  
86 2017). Nonetheless, none of the new HCs reported in previous *in vivo* studies express  
87 Prestin (Chai et al., 2012; Liu et al., 2012a; Walters et al., 2017). Therefore, it is critical  
88 to investigate how Prestin<sup>+</sup> OHCs can be regenerated from SCs, particularly from adult  
89 SCs, in the damaged cochlea. Because ectopic *Ikzf2* in IHCs causes ectopic Prestin  
90 expression (Chessim et al., 2018), we hypothesized that *Atoh1* and *Ikzf2* would not  
91 only synergistically reprogram adult PCs and DCs into HCs, but also produce new  
92 Prestin<sup>+</sup> OHCs.

93 Here, we constructed compound genetic models that allowed us to conditionally  
94 and simultaneously induce ectopic expression of *Atoh1* and *Ikzf2* in adult SCs  
95 (primarily PCs and DCs), with or without pre-damaging wild-type OHCs. Briefly, our  
96 hypothesis was supported by the results of our comprehensive genetic, transcriptomic,  
97 immunostaining, morphological and fate-mapping analyses. New Prestin+ OHC-like  
98 cells were frequently observed across the entire cochlear duct in the case of pre-  
99 damaged OHCs. To the best of our knowledge, this is the first report of the *in vivo*  
100 generation of Prestin+ OHC-like cells from adult SCs through concurrent expression of  
101 *Atoh1* and *Ikzf2*. Our findings have identified *Atoh1* and *Ikzf2* as potential targets for  
102 regenerating OHCs in hearing-impaired patients in the clinic.

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## 104 **RESULTS**

### 105 **Generation of conditional *Ikzf2*-overexpression genetic mouse model**

106 Our aim was to turn on ectopic expression of *Atoh1* and *Ikzf2* specifically in adult  
107 cochlear PCs and DCs. A transgenic conditional *Atoh1*-overexpression strain, *CAG-*  
108 *Loxp-stop-Loxp-Atoh1\*2xHA+* (briefly, *CAG-LSL-Atoh1+*), in which 2× HA fragments  
109 were tagged at the C-terminus of *Atoh1*, can efficiently drive high *Atoh1* expression, as  
110 we reported previously (Liu et al., 2012a). However, a similar conditional expression  
111 model for *Ikzf2* was not available. Thus, by using the CRISPR/Cas9 gene-targeting  
112 approach (Li et al., 2020a), we first generated the *Rosa26-CAG-Loxp-stop-Loxp-*  
113 *Ikzf2\*3xHA-T2A-Tdtomato/+* knockin mouse model (briefly, *Rosa26-CAG-LSL-*  
114 *Ikzf2+/+*), in which *Ikzf2* was tagged with 3×HA fragments at its C-terminus (Figure 1-

115 figure supplement 1A-C). Southern blotting results showed that there was no random  
116 insertion of donor DNA in the mouse genome (Figure 1-figure supplement 1D-E), and  
117 PCR-genotyping of tail DNA allowed us to readily distinguish between littermates with  
118 wild-type, heterozygous, and homozygous genotypes (Figure 1-figure supplement 1F).

119 Heterozygous and homozygous *Rosa26-CAG-LSL-Ikzf2* mice were healthy and  
120 fertile, and did not display any apparent phenotypes. Upon Cre-mediated recombination,  
121 *Tdtomato* and *Ikzf2* were initially translated together from the same polycistronic  
122 mRNA, and subsequently separated by the 2A peptide (Li et al., 2020b). Once triggered,  
123 the expression of *Ikzf2* and *Tdtomato* was permanent, which enabled genetic fate-  
124 mapping analysis at single-cell resolution. Therefore, we could readily distinguish  
125 potential new OHCs (*Tdtomato*+) derived from adult cochlear SCs (primarily PCs and  
126 DCs in this study) from wild-type OHCs (*Tdtomato*-). Because high-quality  
127 commercial antibodies against neither *Atoh1* nor *Ikzf2* were available for  
128 immunostaining, we used an anti-HA antibody to visualize ectopic *Atoh1* and *Ikzf2*  
129 proteins in *CAG-LSL-Atoh1* and *Rosa26-CAG-LSL-Ikzf2/+* strains, respectively.

130 **Ectopic *Ikzf2* was able to induce Prestin expression in IHCs**

131 Before testing *Ikzf2* expression here as a method to regenerate OHCs from adult  
132 cochlear SCs, we determined whether functional *Ikzf2* was efficiently induced in  
133 *Rosa26-CAG-LSL-Ikzf2/+* mice. Prestin is expressed in OHCs exclusively in wild type  
134 mice (Fang et al., 2012; Liberman et al., 2002; Zheng et al., 2000), but it is ectopically  
135 turned on in IHCs infected with an *Anc80*-virus expressing *Ikzf2* (Chessum et al., 2018).  
136 Therefore, the criterion we used was that Prestin expression must be triggered in IHCs

137 upon crossing *Rosa26-CAG-LSL-Ikzf2/+* with the transgenic strain *Atoh1-CreER+*, in  
138 which Cre activity is restricted to HCs (both IHCs and OHCs) (Chow et al., 2006; Cox  
139 et al., 2012). Both *Atoh1-CreER+* (control group) and *Atoh1-CreER+; Rosa26-CAG-*  
140 *LSL-Ikzf2/+* (experimental group) mice were administered tamoxifen at postnatal day  
141 0 (P0) and P1, and analyzed at P42 (Figure 1A and Figure 1-figure supplement 2). We  
142 also comprehensively analyzed another control strain, *Rosa26-CAG-LSL-Ikzf2/+*, and  
143 we did not detect either HA+ (Ikzf2+) or Tdtomato+ cells in the cochlea in this strain,  
144 which was not described further here.

145 We primarily focused on Prestin, HA (Ikzf2), and Tdtomato expression patterns in  
146 IHCs, although Prestin+ OHCs were also targeted. Neither Prestin nor Tdtomato was  
147 expressed in IHCs of control mice (n = 3) at P42 (Figure 1B-B’’’). By contrast, Prestin  
148 was ectopically expressed in IHCs that were vGlut3+/Tdtomato+ (arrows in Figure 1C-  
149 C’’’ but not IHCs that were vGlut3+/Tdtomato- (asterisks in Figure 1C-C’’’)) in the  
150 experimental mice at P42 (n = 3). All Tdtomato+ IHCs were Prestin+ and all Prestin+  
151 IHCs were Tdtomato+. Quantification revealed that  $30.8\% \pm 4.2\%$ ,  $38.4\% \pm 1.6\%$ , and  
152  $70.7\% \pm 6.0\%$  of IHCs were Prestin+ in basal, middle, and apical turns, respectively, at  
153 P42 (Figure 1D and Figure 1-figure supplement 2); thus, the apical turn harbored more  
154 Prestin+ IHCs than the basal and middle turns, in accord with the higher Cre efficiency  
155 of *Atoh1-CreER+* in the apex (Cox et al., 2012). We also confirmed that all Tdtomato+  
156 (Prestin+) IHCs expressed HA (Ikzf2) and vice versa (Figure 1E and F), which  
157 validated the co-expression of Ikzf2 and Tdtomato. This result also suggested that Ikzf2  
158 derepressed Prestin expression in IHCs in a cell-autonomous manner. Moreover,

159 Tdtomato+ OHCs were expected to express both endogenous and ectopic *Ikzf2*, but the  
160 OHCs appeared normal, which suggests that these cells can tolerate additional *Ikzf2*  
161 expression until at least P42 (Figure 1C-C'' and Figure 1F).

162 Together, our results showed that the expression level of *Ikzf2* derived from the  
163 *Rosa26-CAG-LSL-Ikzf2/+* strain was sufficiently high to drive Prestin expression in  
164 IHCs. These Tdtomato+/*Ikzf2*+/Prestin+ IHCs were expected to be distinct from wild-  
165 type IHCs, because these cells might partially transdifferentiate into OHCs or at least  
166 express OHC genes (Chessum et al., 2018). To summarize, we confirmed that *Rosa26-*  
167 *CAG-LSL-Ikzf2/+* was a powerful genetic model suitable for inducing functional *Ikzf2*  
168 expression in adult cochlear SCs.

169 ***Ikzf2* alone failed to convert adult PCs and DCs into HCs**

170 We next determined whether *Ikzf2* alone can reprogram adult cochlear SCs into  
171 Prestin+ OHCs. No Tdtomato+ HCs (neither IHCs nor OHCs) were observed in *Fgfr3-*  
172 *iCreER+; Ai9/+* (briefly, *Fgfr3-Ai9*) mice when tamoxifen was administered at  
173 P30/P31 and the analysis was performed at P60 (arrows in Figure 2A-A''). However,  
174 most SCs (primarily PCs and DCs) were Tdtomato+ (inset in Figure 2A'), which agreed  
175 with our previous reports (Liu et al., 2012a; Liu et al., 2012b). We also confirmed that  
176 *Atoh1* alone failed to convert adult cochlear SCs into HCs in the *Fgfr3-iCreER+; CAG-*  
177 *LSL-Atoh1+* model (briefly, *Fgfr3-Atoh1*) (Figure 2B-B''), same as we reported  
178 previously (Liu et al., 2012a).

179 As expected, numerous Tdtomato+ cells were observed in cochleae of *Fgfr3-*  
180 *iCreER+; Rosa26-CAG-LSL-Ikzf2/+* (briefly, *Fgfr3-Ikzf2*) mice that were given

181 tamoxifen at P30/P31 and analyzed at P60, in both the HC layer (Figure 2C-C'') and  
182 the SC layer (Figure 2D-D''). Again, all Tdtomato+ cells expressed HA and vice versa  
183 (arrows in Figure 2C-D''). However, none of these Tdtomato+/HA+ cells expressed  
184 Prestin (arrows in Figure 2C-D''). Furthermore, we also did not detect any  
185 Tdtomato+/Myo6+ cells. We noted the loss of endogenous OHCs (Prestin+/Tdtomato-)  
186 throughout the cochlear duct, particularly at the basal turn, which was likely a  
187 secondary effect of ectopic *Ikzf2* expression in adult cochlear SCs. Collectively, these  
188 results suggested that *Ikzf2* alone was not sufficient for converting adult cochlear SCs  
189 into nascent Myo6+ HCs or Prestin+ OHCs. Thus, in terms of cell-fate conversion,  
190 more barriers might exist between adult cochlear SCs and OHCs than between IHCs  
191 and OHCs.

192 ***Ikzf2* and *Atoh1* together converted, at low efficiency, adult PCs and DCs into  
193 Prestin+ OHC-like cells**

194 Considering the synergistic effects reported among multiple TFs such as *Six1*,  
195 *Atoh1*, *Pou4f3*, and *Gata3* and between *Myc* and *Notch* signaling (Costa et al., 2015;  
196 Menendez et al., 2020; Shu et al., 2019; Walters et al., 2017), we hypothesized that  
197 concomitant induction of *Ikzf2* and *Atoh1* might convert adult cochlear SCs into OHCs.  
198 We tested this by analyzing *Fgfr3-iCreER+*; *CAG-LSL-Atoh1+*; *Rosa26-CAG-LSL-*  
199 *Ikzf2/+* mice (briefly, *Fgfr3-Atoh1-Ikzf2*) that were given tamoxifen at P30/P31 and  
200 analyzed at P60. Again, Tdtomato+ SCs were abundant within the OC, and 88.0%  $\pm$   
201 2.7%, 94.1%  $\pm$  4.3%, and 98.2%  $\pm$  1.8% of HA+ cells were Tdtomato+ in the basal,  
202 middle, apical turns, respectively (n = 3). The finding that most of the Tdtomato+ cells

203 were HA+ further confirmed the high Cre activity in the *Fgfr3-iCreER* model. The  
204 small fraction of HA+/Tdtomato- cells represented populations in which ectopic Atoh1  
205 but not *Ikzf2* was expressed due to independent Cre-recombination events in the two  
206 loci. Conversely, no Tdtomato+/HA- cells were observed because Tdtomato and HA  
207 are tightly paired in the *Rosa26-CAG-LSL-Ikzf2*/+ model.

208 Unlike in the case of *Ikzf2* induction alone (Figure 2C-D’’’), Tdtomato+/Prestin+  
209 cells were occasionally observed in *Fgfr3-Atoh1-Ikzf2* mice (n = 3) at P60 (arrows in  
210 Figure 2E-F’’’). These cells were defined as new OHC-like cells in our study because  
211 they were derived from the original Tdtomato+ cochlear SCs (PCs and DCs) but were  
212 not identical to wild-type adult OHCs yet. These OHC-like cells were distributed in  
213 both the HC layer (Figure 2E-E’’’) and the SC layer (Figure 2F-F’’’). However, the  
214 numbers of new OHC-like cells detected were only  $4.7 \pm 4.2$ ,  $0.7 \pm 0.3$ , and  $14.3 \pm 6.4$   
215 throughout the entire basal, middle, and apical turns, respectively (Figure 2G).  
216 Moreover, Prestin protein expression was substantially lower in these OHC-like cells  
217 (arrows in Figure 2E-F’’’) than in wild-type endogenous OHCs, which expressed  
218 Prestin but not Tdtomato (arrowheads in Figure 2F-F’’’). Again, endogenous OHC loss  
219 occurred in all cochlear turns (Figure 2E-F’’’). Collectively, these results supported the  
220 conclusion that *Atoh1* and *Ikzf2* together reprogrammed, albeit at low efficiency, adult  
221 cochlear SCs into Prestin+ OHC-like cells, but neither gene alone triggered this  
222 conversion (Figure 2H). Therefore, we next sought to test whether pre-damaging OHCs  
223 would boost the reprogramming efficiency and generate increased numbers of Prestin+  
224 OHC-like cells.

225 **Generation of *Prestin-DTR/+* model for OHC damage**

226 To damage adult OHCs *in vivo*, we used genetic and pharmacological approaches.

227 The diphtheria toxin (DT)/DT receptor (DTR) system has been successfully used to

228 damage HCs in the inner ear (Cox et al., 2014; Golub et al., 2012; Tong et al., 2015).

229 Thus, we generated a new knockin mouse model, *Prestin-P2A-DTR/+* (briefly, *Prestin-*

230 *DTR/+*), in which the P2A-DTR fragment was inserted immediately before the stop

231 codon (*TAA*) of *Prestin* (Figure 3-figure supplement 1A-C). DTR expression was

232 entirely controlled by the endogenous *Prestin* promoter and/or enhancers and restricted

233 to OHCs, and *Prestin* expression itself was intact. Southern blotting results confirmed

234 the absence of random insertion of donor DNA in the genome (Figure 3-figure

235 supplement 1D and E), and tail-DNA PCR allowed us to readily distinguish between

236 wild-type *Prestin* and post-gene-targeting alleles (Figure 3-figure supplement 1F).

237 In the absence of DT treatment, co-staining for the OHC-marker *Prestin* and IHC-

238 marker vGlut3 revealed that OHCs were normal in *Prestin-DTR/+* mice (n = 3) at P42

239 (Figure 3A and A'). By contrast, after a single injection of DT (20 ng/g, body weight)

240 at P36, severe OHC loss was observed in *Prestin-DTR/+* mice (n = 3) at P42, and only

241 very few OHCs were sporadically detected throughout the cochlear duct (arrowhead in

242 Figure 3B'). The debris of dying OHCs were frequently observed at P42 (arrows in

243 Figure 3B') but disappeared by P60 (further described below; see Figure 4). Conversely,

244 IHCs appeared normal at P42 under DT treatment, which confirmed that DTR was

245 specifically expressed in OHC and absent in IHCs (Figure 3B and B'). Furthermore,

246 the results of auditory brainstem response (ABR) measurement demonstrated that the

247 thresholds at distinct frequencies in *Prestin-DTR/+* mice (n=3) treated with DT were  
248 significantly higher than those in control *Prestin-DTR/+* mice (n=3) not treated DT  
249 (Figure 3C). Accordingly, the ratio of OHC numbers to IHC number was ~3.23 in  
250 control (blue in Figure 3D), but was significantly decreased, to ~0.19, in *Prestin-DTR/+*  
251 mice treated with DT (red in Figure 3D). Together, these results showed that DT  
252 treatment caused, within 6 days, marked hearing impairment due to OHC loss, and  
253 further indicated that *Prestin-DTR/+* represented a powerful mouse model for  
254 specifically damaging wild-type OHCs.

255 **Conversion of adult cochlear SCs into Prestin+ OHC-like cells by *Ikzf2* and *Atoh1*  
256 was considerably enhanced when endogenous OHCs were damaged**

257 In nonmammalian vertebrates, HC loss triggers the regeneration of HCs through a  
258 cell-fate change in SCs (Janesick and Heller, 2019; Stone and Cotanche, 2007; Warchol  
259 and Corwin, 1996). Therefore, we tested whether the damage of endogenous wild-type  
260 adult OHCs coupled with the ectopic expression of *Ikzf2* and *Atoh1* in adult cochlear  
261 SCs would efficiently generate OHC-like cells; for this, we used four genetic models:  
262 (1) *Prestin-DTR/+*; (2) *Fgfr3-iCreER+; CAG-LSL-Atoh1+; Prestin-DTR/+ (Fgfr3-*  
263 *Atoh1-DTR)*; (3) *Fgfr3-iCreER+; Rosa26-CAG-LSL-Ikzf2/+; Prestin-DTR/+ (Fgfr3-*  
264 *Ikzf2-DTR)*; and (4) *Fgfr3-iCreER+; CAG-LSL-Atoh1+; Rosa26-CAG-LSL-Ikzf2/+; Prestin-DTR/+ (Fgfr3-Atoh1-Ikzf2-DTR)*. We first turned on dual expression of *Atoh1*  
265 or *Ikzf2* or both (by injecting tamoxifen at P30 and P31) and then triggered OHC  
266 damage 6 days later (by DT treatment at P36) (Figure 4A and B). This strategy allowed  
267 us to precisely and permanently label adult SCs (mainly PCs and DCs) with HA and

269 Tdtomato before OHC damage and thus facilitated the subsequent fate-mapping  
270 analysis for determining whether Prestin+ OHC-like cells were produced. The reverse  
271 order of the experimental procedure was not used so as to avoid the possibility of OHC  
272 damage leading to changes in the Cre-expression pattern in *Fgfr3-iCreER*+ mice.

273 Unlike in control *Prestin-DTR*/+ mice not treated with DT, in which three well-  
274 aligned rows of Prestin+ OHCs were observed at P60 (Figure 4C), only a few OHCs  
275 were occasionally detected in *Prestin-DTR*/+ mice treated DT (arrows in Figure 4D).

276 Moreover, in contrast to the case at P42 (arrows in Figure 3B'), we detected no debris  
277 of dying OHCs at P60 (Figure 4D). Furthermore, no HA+/Prestin+ cells were identified  
278 in either *Fgfr3-Atoh1-DTR* mice (Figure 4E) or *Fgfr3-Ikzf2-DTR* mice (Figure 4F) at  
279 P60, which suggested that damaging wild-type OHCs did not promote production of  
280 the Prestin+ new OHC-like cells when Atoh1 or Ikzf2 was overexpressed alone in adult  
281 cochlear SCs.

282 Conversely, Tdtomato+/HA+/Prestin+ OHC-like cells were frequently observed in  
283 *Fgfr3-Atoh1-Ikzf2-DTR* mice at P60. Confocal scanning of the entire cochlear duct and  
284 quantification (n = 3) revealed the presence of  $359.3 \pm 46.2$ ,  $878.0 \pm 118.7$ , and  $1195 \pm$   
285 81.6 Tdtomato+/HA+ cells in the basal, middle, and apical turns, respectively, among  
286 which  $86.3 \pm 6.3$ ,  $241.0 \pm 21.1$ , and  $190.3 \pm 31.1$  cells were the new OHC-like cells  
287 (arrows in Figure 4G-G''). By normalizing the numbers of OHC-like cells against the  
288 total number of Tdtomato+/HA+ cells per sample, we found that  $25.0\% \pm 4.1\%$ ,  $29.0\%$   
289  $\pm 5.7\%$ , and  $16.2\% \pm 3.4\%$  of adult cochlear SCs expressing both Ikzf2 and Atoh1  
290 transformed into OHC-like cells in the basal, middle, and apical turns, respectively

291 (Figure 4H). Comparison with the results obtained for *Fgfr3-Atoh1-Ikzf2* mice at P60  
292 (Figure 2E-G) revealed not only a significant increase in the number of Prestin+ OHC-  
293 like cells at each cochlear turn in *Fgfr3-Atoh1-Ikzf2-DTR* mice (Figure 4I), but also a  
294 general elevation of Prestin expression in individual cells (Figure 4G-G’’’). However,  
295 the Prestin levels in these OHC-like cells were considerably lower than those in wild-  
296 type OHCs (Figure 4C vs Figure 4G’’). Together, our results demonstrated that  
297 damaging endogenous OHCs markedly enhanced the reprogramming efficiency of  
298 *Ikzf2* and *Atoh1*, and thus considerably increased numbers of OHC-like cells derived  
299 from adult cochlear SCs.

300 **Initial cell-fate transition from SCs to general HCs was followed by a second  
301 switch from general HCs to OHC-like cells**

302 We next determined when these adult cochlear SC-derived OHC-like cells or  
303 nascent new HCs emerged (Figure 4-figure supplement 1A-C’’’). Nascent new HCs  
304 were *Tdtomato*+/*Myo6*+ but had not turned on Prestin expression yet (arrowheads in  
305 Figure 4-figure supplement 1B-C’’’); P42 was the earliest age at which the nascent new  
306 HCs were detected, and *Myo6* expression was weak at this stage. Scanning of the entire  
307 cochlear duct at P42 and quantification revealed the presence of only  $29.0 \pm 13.5$ ,  $66.0$   
308  $\pm 35.23$ , and  $52.7 \pm 25.4$  nascent new HCs throughout the basal, middle, and apical  
309 turns ( $n=3$ ), respectively (black in Figure 4-figure supplement 1D). However, no  
310 *Tdtomato*+/Prestin+ OHC-like cells were detected at P42.

311 Four days later, at P46 ( $n = 4$ ), there were  $56.5 \pm 28.8$ ,  $118.0 \pm 61.2$ , and  $111.0 \pm$   
312  $57.3$  new HCs that were *Tdtomato*+/*Myo6*+ (gray in Figure 4-figure supplement 1D

313 and E), and, of these,  $27.8 \pm 15.0$ ,  $43.5 \pm 25.3$ , and  $36.8 \pm 21.0$  were  
314 Tdtomato+/Myo6+/Prestin+ (OHC-like cells; green in Figure 4-figure supplement 1E);  
315 thus, OHC-like cells accounted for 49.2% (27.8/56.5), 36.9% (43.5/118), and 33.2%  
316 (36.8/111) of total new HCs in basal, middle, and apical turns, respectively. The  
317 remaining Tdtomato+/Myo6+/Prestin- cells were defined as nascent HCs, and the  
318 Tdtomato+/Myo6-/Prestin- cells were defined as SCs that failed to become HCs. Here,  
319 we sorted new HCs into nascent HCs and OHC-like cells based solely on absence or  
320 presence of Prestin. Among the 4 mice analyzed, 2 mice harbored substantially fewer  
321 Tdtomato+/Myo6+ cells, which caused the large variations in numbers; nevertheless,  
322 the overall trend was that the higher the number of Tdtomato+/Myo6+ cells observed,  
323 the higher the number of OHC-like cells observed. Notably, we did not detect even a  
324 single Tdtomato+/Prestin+ cell not expressing Myo6. Together, these findings  
325 suggested that the generation of new OHC-like cells generally involved an initial cell-  
326 fate transition from SCs (PCs and DCs) into nascent HCs by P42 (12 days after turning  
327 on *Atoh1* and *Ikzf2*, and 6 days after DT treatment), and this was followed by a second  
328 transition from nascent HCs into OHC-like cells.

### 329 **Hair bundles were present in the new OHC-like cells**

330 Scanning electron microscopy (SEM) was used to examine the hair bundles (or  
331 stereocilia) of the Prestin+ new OHC-like cells at P60 (Figure 5). The staircase-shaped  
332 hair bundles are the sites where mechanoelectrical transduction (MET) channels are  
333 distributed and are critical for hearing (Corey and Holt, 2016; Douguet and Honore,  
334 2019; Wu and Muller, 2016). The regular V- or W-shaped hair bundles were present in

335 wild-type OHCs from *Prestin-DTR/+* mice not treated with DT (control) (Figure 5A  
336 and A'), whereas very few hair bundles remained in *Prestin-DTR/+* mice treated with  
337 DT (Figure 5B). This agreed with the results of our immunostaining assays (Figure 4D).

338 Intriguingly, we frequently detected stereocilia with a single long bundle but  
339 lacking the staircase shape in *Fgfr3-Atoh1-Ikzf2-DTR* mice at P60 (Figure 5C-C').

340 Stereocilia with several long bundles were seldom observed (inset in Figure 5C). These  
341 are likely the hair bundles of OHC-like cells (or nascent HCs), because such hair  
342 bundles were not observed in *Prestin-DTR/+* mice treated with or without DT (Figure

343 5A-B). ABR measurement results showed that the thresholds at distinct frequencies  
344 were markedly higher in *Fgfr3-Atoh1-Ikzf2-DTR* mice (n=6, red line in Figure 5D) than  
345 in *Prestin-DTR/+* mice without DT treatment (n=3, black line in Figure 5D). However,

346 no significant hearing improvement (lowering of threshold) was recorded between  
347 *Fgfr3-Atoh1-Ikzf2-DTR* and *Prestin-DTR/+* mice upon DT treatment (n=5, blue line in  
348 Figure 5D). Notably, loss of IHCs were observed at P60 (asterisks in Figure 5B and C).

349 It should be a secondary effect caused by OHC death or disruption of the OC structure  
350 because no IHC death was observed at P42 (Figure 3). Collectively, these results  
351 showed that the new OHC-like cells did not yet completely resemble wild-type OHCs,  
352 at least in terms of hair-bundle structure and Prestin expression. The extent to which  
353 OHC-like cells were similar to wild-type OHCs was next addressed using single-cell  
354 RNA-Seq analysis.

355 **Single-cell RNA-Seq revealed unique genes enriched in adult wild-type OHCs and**  
356 **SCs**

357 To perform single-cell RNA-Seq on adult OHCs and SCs, we manually picked 17  
358 wild-type Tdtomato+ OHCs from *Prestin-CreER/+; Ai9/+* mice at P30, and 16 wild-  
359 type Tdtomato+ SCs (primarily PCs and DCs) from *Fgfr3-iCreER+; Ai9/+* mice at P60  
360 (Figure 6A). All cells were identified based on their endogenous Tdtomato fluorescence  
361 and were picked and washed thrice under a fluorescence microscope before final  
362 collection in PCR tubes, after which RNA-Seq libraries were prepared using the Smart-  
363 Seq approach (smartseq) (Figure 6A). Manual picking combined with smartseq has  
364 been successfully used in previous gene-profiling studies on adult cochlear HCs and  
365 SGNs (Li et al., 2020a; Liu et al., 2014; Shrestha et al., 2018). Because smartseq  
366 involves full-length cDNA sequencing, the method provides higher gene coverage than  
367 10 $\times$  genomic single-cell RNA-Seq, which only detects the 3'-end of coding genes (Li  
368 et al., 2020a; Petitpre et al., 2018; Shrestha et al., 2018; Sun et al., 2018; Yamashita et  
369 al., 2018).

370 We first compared gene profiles between wild-type OHCs at P30 (P30\_WT OHCs)  
371 and wild-type SCs at P60 (P60\_WT SCs), and we identified 1324 and 2344 genes  
372 enriched respectively in adult OHCs and adult SCs (Figure 6-figure supplement 1A);  
373 Supplemental File 1 contained the entire list of enriched genes. The OHC-enriched  
374 genes included genes that are recognized to be highly expressed in adult OHCs, such  
375 as *Myo7a*, *Ocm*, *Prestin* (*Slc26a5*), *Ikzf2*, *Espn*, *Tmc1*, *Cib2*, *Lhfp15*, *Lmo7*, *Lbh*, and  
376 *Sri* (Chessum et al., 2018; Du et al., 2019; Giese et al., 2017; Liu et al., 2014; Ranum  
377 et al., 2019; Xiong et al., 2012; Zheng et al., 2000). As expected, previously identified  
378 IHC-specific genes such as *Slc7a14* (blue arrows in Figure 6-figure supplement 1A)

379 were either not detected or minimally detected in our picked Tdtomato+ OHCs (Liu et  
380 al., 2014); by contrast, previously known pan-SC markers, such as *Sox2* and *Sox10*, and  
381 two recently identified DC-specific genes, *Bace2* and *Ceacam16*, were included among  
382 the SC-enriched genes (Li et al., 2018; Ranum et al., 2019). Gene Ontology (GO)  
383 enrichment analysis was performed on the OHC- or SC-enriched genes (Figure 6-figure  
384 supplement 1B and C); the results confirmed that the OHC-enriched genes were  
385 involved in sensory perception of sound, inner ear morphogenesis, neurotransmitter  
386 secretion, and stereocilium organization (Figure 6-figure supplement 1B), and that the  
387 SC-enriched genes were involved in functions such as lipid metabolic process,  
388 regulation of cell shape, and actin cytoskeleton organization (Figure 6-figure  
389 supplement 1C). This agreed with the finding that PCs are critical for the formation of  
390 the tunnel of Corti featuring a unique morphology. The OHC- and SC-enriched gene  
391 lists of each GO category were summarized in Supplemental Files 2 and 3, respectively.  
392 Overall, the results showed that the picked adult wild-type OHCs and SCs were pure  
393 and that our single-cell RNA-Seq data were of high quality. Therefore, these genes  
394 specifically enriched in adult wild-type OHCs and SCs served as references in our  
395 characterization of the new HCs, particularly the OHC-like cells.

396 Although *Myo6* protein is known to be enriched in OHCs, *Myo6* mRNA was not  
397 significantly enriched in adult OHCs, because, in our hands, the mRNA was also  
398 detected (albeit at a lower level) in adult SCs. *Myo6* mRNA is also detected in other  
399 non-HC populations (Kolla et al., 2020; Scheffer et al., 2015). Here, *Myo7a* was  
400 significantly enriched in adult OHCs (red arrows in Figure 6-figure supplement 1A)

401 and also highly expressed in nascent HCs and OHC-like cells, but not in SCs that failed  
402 to become HCs (Figure 6B-C’’’). Therefore, we used *Myo7a* as an early HC marker to  
403 define the general HC fate in the RNA-Seq analysis described below.

404 **OHC-like cells globally upregulated wild-type adult OHC-enriched genes and**  
405 **downregulated adult SC-enriched genes**

406 We next focused on characterizing OHC-like cells and determining the degree to  
407 which these were divergent from the original wild-type adult cochlear SCs. We  
408 manually picked 42 Tdtomato+ cells from *Fgfr3-Atoh1-Ikzf2-DTR* mice at P60 (Figure  
409 6A) and sorted the cells into three types: (1) Tdtomato+/Myo7a+/Prestin+ cells (OHC-  
410 like cells, arrows in Figure 6C-C’’’; n = 11 cells); (2) Tdtomato+/Myo7a+/Prestin- cells  
411 (nascent HCs, asterisks in inset of Figure 6C-C’’’; n = 16); and (3) Tdtomato+/Myo7a-  
412 /Prestin- cells (defined as SCs that failed to become HCs, arrowheads in Figure 6C-C’’’;  
413 n = 15). The proportion of OHC-like cells was 26.2% (11/42), which agreed with the  
414 calculation (16.2%–29.0%) from immunostaining assays (Figure 4H). The result  
415 further validated the suitability of this criterion for our analysis. Moreover, expression  
416 of *Atoh1* and *Ikzf2* (arrows in Figure 6-figure supplement 2A) was enriched in all 42  
417 cells, but not in wild-type adult SCs, which also confirmed that *Atoh1* and *Ikzf2* were  
418 permanently overexpressed in the 42 Tdtomato+ cells (regardless of their cell fates).

419 The results of both UMAP (uniform manifold approximation and projection)  
420 analysis and Pearson correlation-coefficient analysis demonstrated that, as compared  
421 with SCs that failed to become HCs, OHC-like cells were generally more divergent  
422 from adult wild-type SCs and convergent with adult wild-type OHCs (Figure 6D and

423 E). Besides *Prestin* (*Slc26a5*) and *Myo7a* (arrows in Figure 6-figure supplement 2A),  
424 1737 genes were expressed at a significantly higher level in OHC-like cells than in adult  
425 SCs. GO analysis of these 1737 genes revealed that the genes were enriched in functions  
426 involved in sound perception, cell differentiation, and inner ear development (Figure 6-  
427 figure supplement 2B), which further supported the notion that OHC-like cells globally  
428 behave like HCs. Notably, 824 out of the 1737 genes overlapped with genes enriched  
429 in wild-type adult OHCs, such as *Myo7a*, *Pvalb*, *Calb1*, *Rbm24*, *Cib2*, and *Lhfpl5*  
430 (arrows in Figure 6-figure supplement 2A). Conversely, 900 genes were expressed at  
431 significantly higher levels in adult wild-type SCs than in OHC-like cells, and 520 out  
432 of the 900 genes overlapped with adult wild-type SC-enriched genes, such as *Ceacam16*,  
433 *Bace2*, *Tuba1b*, *Gjb2*, and *Rorb* (arrows in Figure 6-figure supplement 2A).  
434 Supplemental File 4 contained the entire list of genes that were differently expressed in  
435 OHC-like cells and adult wild-type SCs, as well as the genes that overlapped with adult  
436 wild-type OHC- or SC-enriched genes.

437 In our examination of the difference between OHC-like cells and adult SCs, we  
438 also included nascent HCs and SCs that failed to become HCs as references for  
439 intermediate cell types. As expected, the identified genes were generally either not or  
440 only slightly unregulated/downregulated in SCs that failed to become HCs (Figure 6-  
441 figure supplement 2). Unexpectedly, however, we found that OHC-like cells and  
442 nascent HCs were similar to each other overall, and these were intermingled in the  
443 Pearson correlation-coefficient analysis (Figure 6E). This finding highlighted the  
444 heterogeneous gene-expression profiles among OHC-like cells and nascent HCs. Thus,

445 although *Prestin* expression was detected in OHC-like cells but not in nascent HCs, the  
446 expression patterns of other HC genes might be the opposite. This possibility was  
447 partially supported by the finding that not all *Prestin*<sup>+</sup> OHC-like cells expressed *Rbm24*,  
448 *Pvalb*, and *Calb1*, according to the results of both RNA-Seq and immunostaining assays  
449 (Figure 6-figure supplement 2 and Figure 6-figure supplement 3). *Rbm24*, *Pvalb*, and  
450 *Calb1* are early pan-HC markers and are normally turned on earlier than *Prestin* in wild-  
451 type OHCs (Grifone et al., 2018; Li et al., 2018; Liu et al., 2012a). Together, our results  
452 showed that OHC-like cells/nascent HCs, relative to SCs that failed to become HCs,  
453 were considerably more similar (but not identical) to adult wild-type OHCs. We next  
454 determined the developmental status of the wild-type OHCs to which the OHC-like  
455 cells were most similar.

456 **OHC-like cells were most similar to wild-type differentiating OHCs at neonatal  
457 ages**

458 By reanalyzing raw data from a recent single-cell RNA-Seq study covering wild-  
459 type cochlear OHCs, IHCs, SCs, GER (greater epithelial ridge) cells, and LER (lesser  
460 epithelial ridge) cells (Kolla et al., 2020), we initially sorted out 87 OHCs at E16, 170  
461 OHCs at P1, and 39 OHCs at P7 (Figure 6-figure supplement 4A). Because OHCs were  
462 highly heterogeneous due to the developmental basal-to-apical, medial-to-lateral  
463 gradient in the cochlear duct, trajectory analysis by using Monocle was applied to  
464 OHCs at the three aforementioned ages (Figure 6-figure supplement 4B and B').  
465 Ultimately, we selected 59/87, 118/170, and 37/39 OHCs at E16, P1, and P7,  
466 respectively, and these cells either represented the majority or were located in the center

467 of the cell populations at each age (within dotted lines in Figure 6-figure supplement 4  
468 B). Lastly, the selected OHCs at the three ages were pooled with OHC-like cells,  
469 nascent HCs, P30\_WT OHCs, and P60\_WT SCs. Five main clusters were identified  
470 (Figure 6F): 10/11 OHC-like cells, 13/16 nascent HCs, and the majority of wild-type  
471 OHCs at P1 (P1\_WT OHCs) belonged to cluster 1 (Figure 6G), whereas 1/11 OHC-like  
472 cells, 3/16 nascent HCs, and wild-type OHCs at E16 (E16\_WT OHCs) formed cluster  
473 2, which was close to cluster 5 (P60\_WT SCs). Wild-type OHCs at P7 (P7\_WT OHCs)  
474 and P30\_WT OHCs formed cluster 3, which suggested that P7 OHCs were well  
475 differentiated (Jeng et al., 2020), and cluster 4 contained a small fraction of P1\_WT  
476 OHCs. Thus, we concluded that OHC-like cells and nascent HCs were not distinguished  
477 at the transcriptomic level, and that these cells together were most similar to P1\_WT  
478 OHCs. This conclusion was further supported by the presence of *Insm1* mRNA and  
479 protein in OHC-like cells (Figure 6-figure supplement 2A and Figure 6-figure  
480 supplement 3A-B’’’). *Insm1* is only transiently expressed in differentiating OHCs at  
481 late embryonic or perinatal ages in a basal-to-apical gradient (Lorenzen et al., 2015).

#### 482 **OHC-like cells were considerably less differentiated than adult wild-type OHCs**

483 The finding that OHC-like cells were most similar to P1\_WT OHCs led us to  
484 further compare the transcriptomic profiles between OHC-like cells and P30\_WT  
485 OHCs and thus determine the main differences in their molecular signatures (Figure 6-  
486 figure supplement 5A). Here, the expression of *Atoh1*, but not *Ikzf2*, was higher in  
487 OHC-like cells than in P30\_WT OHCs (Figure 6-figure supplement 5A); this was  
488 because *Atoh1* is not expressed in adult OHCs but *Ikzf2* is (Chessum et al., 2018; Liu

489 et al., 2014). Besides *Insm1*, *Hes6* was enriched in OHC-like cells (Figure 6-figure  
490 supplement 5A). Two previous reports have suggested that *Hes6* is expressed in  
491 cochlear HCs and is a target of Atoh1 (Qian et al., 2006; Scheffer et al., 2007). We also  
492 noted that adult wild-type SC-enriched genes such as *Fgfr3*, *Id2*, and *Id3* were  
493 expressed at higher levels in OHC-like cells than in P30\_WT OHCs (Figure 6-figure  
494 supplement 5A); this was because these SC-enriched genes were not drastically  
495 downregulated in OHC-like cells. In Supplemental File 6, we summarized the entire  
496 list of genes differently expressed in OHC-like cells and P30\_WT OHCs. GO analysis  
497 results showed that genes expressed at higher levels in OHC-like cells were enriched in  
498 cell adhesion, angiogenesis, and neuron projection development (Figure 6-figure  
499 supplement 5B). The enrichment of neural developmental genes in OHC-like cells was  
500 as expected: these genes are transiently expressed in wild-type differentiating HCs, but  
501 are gradually repressed by *Gfi1* in mature HCs (Matern et al., 2020). The complete gene  
502 list of each GO category was summarized in Supplemental File 7.

503 Conversely, genes such as *Ocm*, *Prestin* (*Slc26a5*), *Lmo7*, and *Tmc1* were  
504 expressed at a lower level in OHC-like cells than in P30\_WT OHCs (Figure 6-figure  
505 supplement 5A). These genes were enriched in adult wild-type OHCs (Supplemental  
506 File 1). Notably, *Lmo7* mutant mice have been reported to show abnormalities in HC  
507 stereocilia (Du et al., 2019). GO analysis revealed that the genes expressed at higher  
508 levels in P30\_WT OHCs were enriched in transport and lipid transport (Figure 6-figure  
509 supplement 5C). The complete gene list of each GO category was summarized in  
510 Supplemental File 8.

511 **DISCUSSION**

512 Our *in vivo* study clearly demonstrated the ability of *Ikzf2* and *Atoh1* to effectively  
513 convert adult cochlear SCs (mainly PCs and DCs) into Prestin+ OHC-like cells under  
514 the condition of OHC damage. Because adult cochlear SCs are considerably more  
515 challenging to reprogram than the corresponding neonatal cells, we considered our  
516 work to represent a notable advance in OHC regeneration studies. We expect *Atoh1* and  
517 *Ikzf2* to serve as potential targets for OHC regeneration in the clinic.

518 **Potential roles of *Atoh1* and *Ikzf2* in cell-fate transition from adult cochlear SCs  
519 into OHC-like cells**

520 During normal development of HCs, *Atoh1* is expressed for approximately one  
521 week, and the turning on/off of the expression follows a basal-to-apical gradient. Thus,  
522 the earlier *Atoh1* expression is turned on at a specific location, the earlier it is turned  
523 off. During this period, *Atoh1* is reported to perform at least three age-dependent  
524 functions: specifying the general HC fate, maintaining the survival of HC progenitors  
525 (short-term) and OHCs (long-term), and organizing the HC bundle (Bermingham et al.,  
526 1999; Cai et al., 2013; Woods et al., 2004). Although other functions cannot be ruled  
527 out, we speculated that the role of *Atoh1* was to specify PCs and DCs with a general  
528 HC fate, which was partially evidenced by the inability of *Ikzf2* alone to convert adult  
529 SCs into HCs. However, why *Atoh1* alone was unable to trigger this conversion is  
530 unclear. The detailed synergistic effects between *Atoh1* and *Ikzf2* in the cell-fate  
531 conversion process warrants future investigation.

532 *Ikzf2* is necessary for OHC maturation, and an *Ikzf2* point-mutation model, *Ikzf2*  
533 *cello/cello*, displays early-onset hearing loss and diminished Prestin expression; by contrast,  
534 ectopic *Ikzf2* induces Prestin expression in IHCs (Chessum et al., 2018). Whether  
535 *Prestin* is a direct target of *Ikzf2* is unclear, but the onset of *Ikzf2* expression at neonatal  
536 ages and the specific and permanent expression of the gene in OHCs suggest that *Ikzf2*  
537 is a key TF required to specify and maintain the OHC fate, or to repress the IHC fate.  
538 Therefore, we speculated that the primary role of *Ikzf2* was to direct nascent HCs into  
539 the OHC differentiation track, and, accordingly, *Ikzf2* alone was able to induce Prestin  
540 expression in wild-type IHCs but not adult SCs, partially because IHCs are in the  
541 general HC differentiation track and are poised to turn on OHC genes. We also noted  
542 that the cell-fate switching from adult SCs to HCs induced by *Atoh1* and *Ikzf2* together  
543 occurred in a shorted period (~12 days) than the switching from neonatal SCs to HCs  
544 induced by *Atoh1* alone (~3 weeks) (Liu et al., 2012a). This again might result from the  
545 synergistic actions of *Atoh1* and *Ikzf2*.

546 **547 Transcriptomic difference between Prestin+ OHC-like cells and adult wild-type  
OHCs**

548 A promising advance reported here is our successful *in vivo* conversion of adult  
549 cochlear SCs into OHC-like cells expressing *Prestin*, *Insm1*, and *Ocm*, which are  
550 primarily expressed in OHCs but not IHCs (Lorenzen et al., 2015; Simmons et al., 2010;  
551 Zheng et al., 2000; Zhu et al., 2019); this reprogramming efficiency ranged between  
552 16.2% and 29.0% depending on the different cochlear turns (Figure 4H). OHC-like cells  
553 upregulated 824 genes and downregulated 520 genes enriched in adult wild-type OHCs

554 and SCs, respectively; however, the OHC-like cells differed from the fully  
555 differentiated OHCs present at P30 in both molecular and morphological aspects  
556 (Figures 5 and 6). For the analyses, we selected OHCs at P30 but not P60 because the  
557 OHC-like cells were derived from SCs at P30 and thus their intrinsic age might be ~P30,  
558 although the mice were analyzed at P60. Notably, cochlear development is complete by  
559 P30, and wild-type OHCs at P30 and P60 are expected to differ minimally, a notion  
560 supported at least partly by the finding that adult cochlear SCs at P60 and P120 were  
561 indistinguishable from each other (Hoa et al., 2020).

562 To precisely evaluate the differentiation status of the cells studied here, we also  
563 reanalyzed data from a recent single-cell RNA-Seq study (Kolla et al., 2020). HC  
564 differentiation occurs in a basal-to-apical, medial-to-lateral gradient (Groves et al.,  
565 2013; Wu and Kelley, 2012), and to minimize gene-profiling variations among the  
566 differentiating OHCs at different cochlear turns, we selected OHCs at E16 that were  
567 less differentiated in the trajectory line, and these were assumed to be from the middle  
568 turn (Figure 6-figure supplement 4B). The non-selected OHCs at E16 were expected to  
569 be from the basal turn, and these were close to OHCs at P1. No apical OHCs were  
570 present at E16. Similarly, we selected OHCs at P1 that were distributed in the center of  
571 the trajectory line (Figure 6-figure supplement 4B), and thus we assumed that these  
572 cells were OHCs at P1 at the middle turn. The OHCs at P7 were highly homogenous  
573 and we speculated that these were apical (less differentiated) OHCs that could  
574 effectively tolerate the procedures used for preparing single-cell suspensions.  
575 Accordingly, with the same protocols being used, sequencing data obtained at younger

576 ages were found to be of higher quality than the data from P7, and performing the  
577 analyses at ages after P7 was challenging (Kolla et al., 2020). Our data support the view  
578 that, instead of resembling P30\_WT OHCs, the OHC-like cells were most similar to  
579 P1\_WT OHCs (Figure 6F and G). The age P1 here might be a rough estimate because  
580 more precise gene profiling of OHCs between P1 and P7 is not yet available.

581 **Potential approach to promote OHC-like cells to resemble wild-type adult OHCs**

582 What is the main disparity between the OHC-like cells described here and P30\_WT  
583 OHCs? This is a key question because the final goal is to convert adult cochlear SCs  
584 into functional OHCs that exhibit high Prestin expression levels, overall molecular  
585 signatures, and cellular morphological features similar to those of wild-type adult  
586 OHCs. However, because hearing function was not rescued in this study (Figure 5D),  
587 additional methods must be used to further promote the current Insm1+ OHC-like cells  
588 expressing low levels of Prestin to move forward into the fully differentiated state in  
589 which the cells do not express Insm1 but abundantly express Prestin/Ocm (Lorenzen et  
590 al., 2015; Simmons et al., 2010; Zheng et al., 2000). Our results clearly showed that the  
591 stereocilium structure was one of the main differences between OHC-like cells and  
592 adult OHCs (Figure 5), but the OHC-like cells expressed multiple MET-channel-related  
593 proteins such as Cib2 and Tmc1 (Jia et al., 2020; Li et al., 2019; Pan et al., 2013). This  
594 agrees with the previous report that MET-channel assembly does not require normal  
595 hair-bundle morphology (Cai et al., 2013).

596 In future studies, we aim to focus on discovering additional critical genes such as  
597 *Emx2* that are necessary for hair-bundle organization and growth (Jacobo et al., 2019;

598 Jiang et al., 2017). Instead of the OHC-like cells in our model here that were  
599 heterogeneous in terms of their gene profiles, we will compare neonatal OHCs at P1 in  
600 one particular cochlear duct location with their counterparts at P30; this should allow  
601 the prominent candidate genes that are differently expressed at the two ages to be sorted  
602 in a more efficient manner, and we will then employ our genetic loss-of-function  
603 screening approach (Zhang et al., 2018). Considering the promising results obtained  
604 such as finding the optimal phenotype of the OHC hair bundles being considerably  
605 shorter or more disorganized relative to control, but with the OHCs generated normally  
606 and surviving—we will combine additional candidate genes with *Atoh1* and *Ikzf2*, and  
607 this could lead to the generation of OHC-like cells that show superior differentiation  
608 relative to the cells produced here. In summary, we consider the *in vivo* ability of *Atoh1*  
609 and *Ikzf2* to successfully reprogram adult cochlear SCs into Prestin<sup>+</sup> OHC-like cells in  
610 the presence of OHC damage to be a great advance and highly encouraging. We believe  
611 that *Atoh1* and *Ikzf2* will serve as key targets for future OHC regeneration therapies in  
612 the clinic.

613

## 614 MATERIALS AND METHODS

### 615 Generation of *Rosa26-CAG-LSL-Ikzf2/+* and *Prestin-DTR/+* knockin mouse 616 strains by using CRISPR/Cas9 approach

617 The *Rosa26-CAG-Loxp-stop-Loxp-Ikzf2\*3xHA-T2A-Tdtomato/+* (*Rosa26-CAG-*  
618 *LSL-Ikzf2/+*) knockin mouse strain was produced by co-injecting one sgRNA against  
619 the *Rosa26* locus (5'-ACTCCAGTCTTCTAGAAGA-3'), donor DNA (Figure 1-figure

620 supplement 1), and Cas9 mRNA into one-cell-stage mouse zygotes. A similar strategy  
621 was applied to generate the *Prestin-P2A-DTR/+* (*Prestin-DTR/+*) knockin mouse strain.  
622 The donor DNA is described in Figure 3-figure supplement 1, and the sgRNA against  
623 the *Prestin* (*Slc26a5*) locus was the following: 5'-CGAGGCATAAAGGCCCTGTA-3'.  
624 F0 mice with potential correct gene targeting were screened by performing junction  
625 PCR, and this was followed by crossing with wild-type C57BL/6 mice for germ-line  
626 transition (production of F1 mice). F1 mice were further confirmed by junction PCR.  
627 No random insertion of donor DNAs in the genome of F1 mice was detected in Southern  
628 blotting (Figure 1-figure supplement 1D and E, and Figure 3-figure supplement 1D and  
629 E), performed according to our previously described protocol (Li et al., 2018), and the  
630 two mouse strains were PCR-genotyped using tail DNA (representative gel images of  
631 PCR products were presented in Figure 1-figure supplement 1F and Figure 3-figure  
632 supplement 1F). Detailed primer sequences were described in Supplemental File 9. All  
633 mice were bred and raised in SPF-level animal rooms, and animal procedures were  
634 performed according to the guidelines (NA-032-2019) of the IACUC of Institute of  
635 Neuroscience (ION), CAS Center for Excellence in Brain Science and Intelligence  
636 Technology, Chinese Academy of Sciences.

### 637 **Sample processing, histology and immunofluorescence assays, and cell counting**

638 Adult mice were anesthetized and sacrificed with the heart being perfused with 1×  
639 PBS and fresh 4% paraformaldehyde (PFA) to completely remove blood from the inner  
640 ear, after which inner ear tissues were dissected out carefully, post-fixed with fresh 4%  
641 PFA overnight at 4°C, and washed thrice with 1× PBS. The adult inner ears were first

642 were decalcified with 120 mM EDTA for 2 days at 4°C until they were soft and ready  
643 for micro-dissecting out the cochlear sensory epithelium for use in whole-amount  
644 preparation and immunostaining with the following first antibodies: anti-HA (rat, 1:200,  
645 11867423001, Roche), anti-Prestin (goat, 1:1000, sc-22692, Santa Cruz), anti-Myo6  
646 (rabbit, 1:500, 25-6791, Proteus Bioscience), anti-Myo7a (rabbit, 1:500, 25-6791,  
647 Proteus Bioscience), anti-Sox2 (goat, 1:500, sc-17320, Santa Cruz), anti-Insm1 (guinea  
648 pig, 1:6000; a kind gift from Dr. Shiqi Jia from Jinan University, Guangzhou, China,  
649 and Dr. Carmen Birchmeier from Max Delbrück Center for Molecular Medicine, Berlin,  
650 Germany), anti-Parvalbumin (mouse, 1:500, P3088, Sigma), anti-Rbm24 (rabbit, 1:500,  
651 18178-1-AP, Proteintech), anti-Calbindin (rabbit, 1:500, C9848, Sigma), and anti-  
652 vGlut3 (rabbit, 1:500, 135203, Synaptic System). Cochlear tissues were counterstained  
653 with Hoechst 33342 solution in PBST (1:1000, 62249, Thermo Scientific) to visualize  
654 nuclei, and were mounted with Prolong gold antifade medium (P36930, Thermo  
655 Scientific). Nikon C2, TiE-A1, and NiE-A1 plus confocal microscopes were used to  
656 capture images.

657 Each whole-mount preparation of the cochlear duct was divided into three parts,  
658 which were initially scanned at 10× magnification under a confocal microscope. In the  
659 obtained images, a line was drawn in the middle of IHCs and OHCs to precisely  
660 measure the entire length of each cochlear duct, which was then divided into basal,  
661 middle, and apical portions that were of equal length. For determining the degree of  
662 OHC death in the *Prestin-DTR/+* model (Figure 3), in each mouse, both the OHC and  
663 the IHC numbers in the same scanning regions (60×, confocal microscopy) were

664 quantified at each turn (two different areas were selected and the average number was  
665 calculated); the IHC number was used as a reference because IHCs did not die  
666 following DT treatment. In terms of counting Prestin+ IHCs in *Atoh1-CreER*+/;  
667 *Rosa26-CAG-LSL-Ikzf2*+/ mice (Figure 1D), and Tdtomato+/HA+ cells, OHC-like  
668 cells, and nascent HCs in *Fgfr3-Atoh1-Ikzf2* and *Fgfr3-Atoh1-Ikzf2-DTR* mice (Figs. 2  
669 and 4), the entire cochlear duct of each mouse was scanned using a confocal microscope  
670 (60 $\times$ ) to minimize variation between different replicates. All cell counting data are  
671 presented as means  $\pm$  SEM. Statistical analyses were performed using one-way  
672 ANOVA, followed by a Student's *t* test with Bonferroni correction. GraphPad Prism  
673 6.0 was used for all statistical analyses.

674 **Single-cell RNA-Seq and bioinformatics analysis**

675 Three different models were used to pick Tdtomato+ cells: (1) *Prestin-CreER*+/;  
676 *Ai9*+/ mice that were injected with tamoxifen at P20 and P21; all Tdtomato+ cells were  
677 OHCs at P30, because of exclusive Cre activity in OHCs (Fang et al., 2012). (2) *Fgfr3-*  
678 *iCreER*+/; *Ai9*+/ mice that were administered tamoxifen at P30 and P31; all Tdtomato+  
679 cells within the cochlear sensory epithelium were SCs (primarily PCs and DCs) at P60,  
680 according to our previous reports (Liu et al., 2012a; Liu et al., 2012b). (3) *Fgfr3-Atoh1-*  
681 *Ikzf2-DTR* mice that were administered tamoxifen at P30 and P31 and then DT at P36.  
682 The Tdtomato+ cells within the cochlear sensory epithelium at P60 included OHC-like  
683 cells, nascent HCs, and SCs that failed to become HCs.

684 The cochlear sensory epithelium from each model mouse was carefully dissected  
685 out, digested, and used for preparing single-cell suspensions according to our detailed

686 protocol described previously (Li et al., 2020a). All the aforementioned Tdtomato+  
687 cells were picked under a fluorescence microscope (M205FA, Leica) as described in  
688 Figure 6A. We picked 17 wild-type adult OHCs at P30, 16 wild-type adult SCs at P60,  
689 and 42 Tdtomato+ cells from the *Fgfr3-Atoh1-Ikzf2-DTR* model (Figure 6D), and we  
690 immediately used these cells for reverse-transcription and cDNA amplification with a  
691 Smart-Seq HT kit (Cat# 634437, Takara). The cDNAs (1 ng each) were tagmented using  
692 a TruePrep DNA Library Prep Kit V2 for Illumina (Cat# TD503, Vazyme) and a  
693 TruePrep Index Kit V2 for Illumina (Cat# TD202, Vazyme). The final libraries were  
694 subject to paired-end sequencing on the Illumina Novaseq platform. Each library was  
695 sequenced to obtain 4G raw data.

696 FastQC (v0.11.9) and trimmomatic (v0.39) were used for quality control of raw  
697 sequencing data. For ~70% - 80% of the reads, high-quality mapping to the mouse  
698 reference genome (GRCm38) was achieved by using Hisat2 (v2.1.0) with default  
699 parameters. Raw counts were calculated using HTSeq (v0.10.0), and gene-expression  
700 levels were estimated by using StringTie (v1.3.5) with default parameters. Gene  
701 abundances were presented as transcript per million (TPM) values. Differentially  
702 expressed genes (DEGs) were analyzed using R package “DESeq2” (p.adj < 0.05,  
703 absolute value of (log2 Fold Change) > 2), and the DEGs were used for determining  
704 biological process enrichment (p < 0.05, adjusted using Bonferroni correction) by using  
705 DAVID (Database for Annotation, Visualization and Integrated Discovery). All the raw  
706 data from our single-cell RNA-Seq work have been deposited in the GEO (Gene  
707 Expression Omnibus) under Accession No. GSE161156.

708 Seurat (R package v3.0) was applied to the raw data collected for wild-type OHCs  
709 at E16, P1, and P7 in a recent study (Kolla et al., 2020). To more precisely compare the  
710 transcriptomic profiles obtained from 10 $\times$  genomics and smartseq approaches, we  
711 integrated them first by using the functions “FindIntegrationAnchors” (k.filter = 30)  
712 and “IntegrateData.” Principal components (PCs) were calculated using the “RunPCA”  
713 function, and the top 20 PCs were used for the dimensionality reduction process  
714 (“RunTSNE” and “RunUMAP”). Unsupervised clustering was performed using the  
715 “FindClusters” function (resolution = 0.5). Furthermore, to more precisely define  
716 OHCs (E16, E17, and P7) versus OHC progenitors (E14), we used criteria that were  
717 more stringent than those applied in the previous report. E16\_WT OHCs were defined  
718 as cells in which the individual expression level of *Insm1*, *Myo6*, and *Atoh1* was above  
719 zero; in P1\_WT OHCs, *Bcl11b*, *Myo6*, *Myo7a*, and *Atoh1* expression was above zero;  
720 and in P7\_WT OHCs, *Prestin* (*Slc26a5*), *Myo6*, *Ocm*, and *Ikzf2* expression was above  
721 zero. Trajectory analysis was performed using Monocle (R package v2.0). Pre-  
722 processed Seurat datasets were imported into Monocle by using the “importCDS”  
723 function. The results of differential expression (DE) analysis identified genes that were  
724 significantly altered among OHCs at the three ages. Cells were ordered along a  
725 pseudotime axis by using the “orderCells” function in Monocle.

## 726 **ABR measurement**

727 ABR measurements were performed by using sound at 4k, 5.6k, 8k, 11.3k, 16k,  
728 22.6k, and 32k Hz, according to the detailed protocol described in our previous report  
729 (Li et al., 2018). Student’s *t* test was used for statistical analysis at each frequency

730 between two conditions (Figure 3C and Figure 5D).

731 **Tamoxifen and DT treatment**

732 Tamoxifen (Cat# T5648, Sigma) was dissolved in corn oil (Cat# C8267, Sigma)  
733 and injected intraperitoneally at 3 mg/40 g body weight (P0 and P1) or 9 mg/40 g body  
734 weight (P20 and P21, and P30 and P31). DT (Cat# D0564, Sigma) dissolved in 0.9%  
735 NaCl solution was also delivered through intraperitoneal injection, at a dose of 20 ng/g  
736 body weight, and the mice were analyzed at P42 or P46 or P60.

737 **SEM analysis**

738 SEM was performed following the protocol reported previously (Parker et al.,  
739 2016). Briefly, we made holes at the cochlear apex and then washed the samples gently  
740 with 0.9% NaCl (Cat#10019318, Sinopharm Chemical Reagent Co, Ltd.) and fixed  
741 them with 2.5% glutaraldehyde (Cat# G5882, Sigma) overnight at 4°C. On the  
742 following day, the cochlear samples were washed thrice with 1× PBS and then  
743 decalcified using 10% EDTA (Cat# ST066, Beyotime) for 1 day, post-fixed for 1 h with  
744 1% osmium tetroxide (Cat#18451, Ted Pella), and subject to a second fixation for 30  
745 min with thiocarbohydrazide (Cat#88535, Sigma) and a third fixation for 1 h with  
746 osmium tetroxide. Next, the cochlear samples were dehydrated using a graded ethanol  
747 series (30%, 50%, 75%, 80%, 95%; Cat#10009259, Sinopharm Chemical Reagent Co,  
748 Ltd.), at 4°C with 30 min used at each step, and, lastly, dehydrated in 100% ethanol  
749 thrice (30 min each) at 4°C. The cochlear samples were dried in a critical point dryer  
750 (Model: EM CPD300, Leica), after which whole-mount cochlear samples were  
751 prepared under a microscope to ensure that hair bundles were facing upward and then

752 treated in a turbomolecular pumped coater (Model: Q150T ES, Quorum). The final  
753 prepared samples were scanned using a field-emission SEM instrument (Model:  
754 GeminiSEM 300, ZEISS).

755

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765

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946 **FIGURE LEGENDS**

947 **Figure 1. Prestin was expressed in IHCs when ectopic *Ikzf2* was turned on. (A)**

948 Illustration of how *Ikzf2* expression was turned on in HCs (both IHCs and OHCs).

949 Tdtomato and *Ikzf2* (tagged with HA) were tightly paired. *Atoh1-CreER*<sup>+</sup> is an efficient

950 HC cre driver at neonatal ages. **(B-C'')** Triple labeling for Prestin, Tdtomato, and

951 vGlut3 in P42 cochlear samples: control *Atoh1-CreER*<sup>+</sup> (B-B'') and experimental

952 *Atoh1-CreER*<sup>+</sup>; *Rosa26-CAG-LSL-Ikzf2*<sup>+/+</sup> (C-C''). Prestin was only expressed in

953 wild-type OHCs (B-B''). Arrows in (C-C''): Tdtomato+/vGlut3+/Prestin+ IHC;

954 asterisks in (C-C''): vGlut3+/Tdtomato- IHC that did not turn on Prestin expression in

955 experimental mice. **(D)** Quantification of Prestin+ IHCs. More Prestin+ IHCs were

956 present in the apical turn than in basal and middle turns. \*\* p < 0.01. **(E-F)** Co-staining

957 of HA (*Ikzf2*) and Tdtomato in control (E) and experimental (F) mice at P42.

958 HA+/Tdtomato+ cells were present in experimental mice only. Arrow/asterisk in (F):

959 IHC with/without HA (*Ikzf2*) expression. All Tdtomato+ cells were HA+ (*Ikzf2*-

960 expressing) cells, and vice versa. Scale bars: 20  $\mu$ m.

961

962 **Figure 2. *Atoh1* and *Ikzf2* together converted adult PCs and DCs into OHC-like**

963 **cells at low efficiency.** Triple labeling for HA, Tdtomato, and Prestin in four different

964 mouse genetic models that were administered tamoxifen at P30 and P31 and then

965 analyzed at P60. **(A-A'')** All Tdtomato+ were SCs (primarily PCs and DCs) in *Fgfr3*-

966 *iCreER*<sup>+</sup>; *Ai9*<sup>+/+</sup> (*Fgfr3-Ai9*) mice. Inset in (A'): confocal image scanned at SC layer.

967 Arrows in (A-A''): Prestin+/Tdtomato- OHC. **(B-B'')** No Tdtomato signal was

968 detected in *Fgfr3-iCreER+; CAG-LSL-Atoh1+* (*Fgfr3-Atoh1*) mice, and no  
969 HA+/Prestin+ cells were observed. Inset in (B): confocal image scanned at SC layer.  
970 (**C-D””**) Confocal images scanned at HC layer (C-C”” and SC layer (D-D”” in  
971 cochlear samples from *Fgfr3-iCreER+; Rosa26-CAG-LSL-Ikzf2/+* (*Fgfr3-Ikzf2*) mice.  
972 Arrows in both layers: two cells that were HA+/Tdtomato+ but did not express Prestin.  
973 Alignment of wild-type Prestin+ OHCs was abnormal. (**E-F””**) Confocal images  
974 scanned at HC layer (E-E”” and SC layer (F-F”” in cochlear samples from *Fgfr3-*  
975 *iCreER+; CAG-LSL-Atoh1+; Rosa26-CAG-LSL-Ikzf2/+* (*Fgfr3-Atoh1-Ikzf2*) mice.  
976 According to different cellular morphologies and location, arrows in (E-E”” indicate  
977 an OHC-like cell that was HA+/Tdtomato+/Prestin+ and derived from adult DCs; by  
978 contrast, the arrows in (F-F”” indicate another OHC-like cell derived from adult PCs.  
979 Arrowheads: Prestin+/Tdtomato- wild-type OHC appearing in SC layer. Prestin  
980 expression in the new OHC-like cells was lower than that in wild-type OHCs. (**G**)  
981 Quantification of OHC-like cells throughout entire cochlear turns in the *Fgfr3-Atoh1-*  
982 *Ikzf2* model. Data are presented as means  $\pm$  SEM (n=3). OHC-like cells were  
983 reproducibly observed, but the cell numbers were low and showed large variations. (**H**)  
984 Summary of reprogramming outcomes in the three models studied here. OHC-like cells  
985 were present only when *Atoh1* and *Ikzf2* were concurrently reactivated in adult cochlear  
986 SCs. Scale bars: 20  $\mu$ m.

987  
988 **Figure 3. Damaging adult OHCs specifically by using genetic and pharmacological**  
989 **approaches. (A-B')** *Prestin-DTR/+* mice were treated without (A-A', control) or with

990 (B-B', experimental) diphtheria toxin (DT) at P36 and analyzed at P42. Samples were  
991 co-stained for Prestin and vGlut3. (A') and (B'): magnified images of indicated square  
992 areas in (A) and (B). DT treatment led to rapid OHC death within 6 days and only a  
993 few OHCs remained (arrowhead in B'), and debris of dying OHCs were frequently  
994 detected (arrows in B'). Much of the green signal in (B) was from the debris of dying  
995 OHCs. **(C)** Auditory brainstem response (ABR) measurement of *Prestin-DTR/+* mice  
996 treated without (blue line) or with (red line) DT. ABR thresholds were significantly  
997 higher throughout cochlear ducts after DT treatment. **(D)** Ratios of OHCs to IHCs in  
998 control mice (blue) and experimental mice (red) in the same confocal scanning areas;  
999 OHC numbers were significantly decreased in the experimental mice. \*\* p<0.01, \*\*\*  
1000 p<0.001, \*\*\*\* p<0.0001. Scale bars: 200  $\mu$ m (B), 20  $\mu$ m (B').

1001

1002 **Figure 4. Damaging wild-type OHCs enhanced reprogramming efficiency of**  
1003 **Atoh1 and Ikzf2 in adult SCs. (A)** Identical treatments were applied to the distinct  
1004 genetic mouse models: tamoxifen treatment at P30 and P31, followed by DT treatment  
1005 at P36 and analysis at P60. **(B)** Drawing illustrating key events at the cellular level:  
1006 turning on *Atoh1* and *Ikzf2* in adult PCs and DCs that were also permanently labeled  
1007 with Tdtomato for the subsequent fate-mapping analysis. **(C-G””)** Triple labeling for  
1008 HA, Tdtomato, and Prestin in four models: (1) *Prestin-DTR/+* (C and D), (2) *Fgfr3-*  
1009 *iCreER+; CAG-LSL-Atoh1+; Prestin-DTR/+* (*Fgfr3-Atoh1-DTR*; E), (3) *Fgfr3-*  
1010 *iCreER+; Rosa26-CAG-LSL-Ikzf2/+; Prestin-DTR/+* (*Fgfr3-Ikzf2-DTR*; F), and (4)  
1011 *Fgfr3-iCreER+; CAG-LSL-Atoh1+; Rosa26-CAG-LSL-Ikzf2/+; Prestin-DTR/+*

1012 (Fgfr3-Atoh1-Ikzf2-DTR; G-G’’’). Relative to *Prestin-DTR/+* mice not treated with DT  
1013 (C), DT-treated *Prestin-DTR/+* mice harbored very few normal Prestin+ OHCs at P60  
1014 (arrows in D). Debris of dying OHCs had disappeared. No OHC-like cells were  
1015 observed in the first three models, but Tdtomato+/HA+/Prestin+ OHC-like cells  
1016 (arrows in G-G’’’) were present in the *Fgfr3-Atoh1-Ikzf2-DTR* model. **(H)** Percentages  
1017 of OHC-like cells at different cochlear turns in *Fgfr3-Atoh1-Ikzf2-DTR* mice. **(I)**  
1018 Comparison of OHC-like cell numbers between *Fgfr3-Atoh1-Ikzf2-DTR* and *Fgfr3-*  
1019 *Atoh1-Ikzf2* models (without damaging adult wild-type OHCs). *Fgfr3-Atoh1-Ikzf2-*  
1020 *DTR* mice harbored considerably more OHC-like cells than *Fgfr3-Atoh1-Ikzf2* mice. \*\*  
1021 p<0.01, \*\*\* p<0.001. Scale bars: 20  $\mu$ m.

1022

1023 **Figure 5. Hair bundles were present in OHC-like cells.** Scanning electron  
1024 microscopy (SEM) analysis of samples from three different mouse models at P60. **(A-**  
1025 **A’)** OHCs harbored V- or W-shaped HC bundles in *Prestin-DTR/+* mice not treated  
1026 with DT. (A’): high-magnification view of black rectangle in (A). **(B)** Almost all OHCs  
1027 disappeared in *Prestin-DTR/+* mice upon DT treatment at P36. Black asterisk: one IHC  
1028 that was absent. **(C)** Immature hair bundles were frequently detected in the *Fgfr3-*  
1029 *iCreER+; CAG-LSL-Atoh1+; Rosa26-CAG-LSL-Ikzf2/+; Prestin-DTR/+ (Fgfr3-*  
1030 *Atoh1-Ikzf2-DTR*) model, but not in (A) and (B). These hair bundles were expected to  
1031 originate from OHC-like cells. (C’): high-magnification view of black rectangle in (C).  
1032 Inset in C: HC bundles that were observed relatively less frequently. Black asterisk: one  
1033 IHC that was missing. **(D)** ABR measurements of these three models. Relative to the

1034 threshold in *Prestin-DTR/+* mice not treated with DT (black line), the ABR thresholds  
1035 in *Prestin-DTR/+* treated with DT (blue line) and in *Fgfr3-Atoh1-Ikzf2-DTR* mice (red  
1036 line) were significantly increased. The blue and red lines showed no statistically  
1037 significant difference at any frequency (n.s.). \*\* p<0.01, \*\*\*\* p<0.0001. Scale bars: 5  
1038  $\mu\text{m}$  (A, B, C), 1  $\mu\text{m}$  (C'), and 500 nm (A').

1039

1040 **Figure 6. OHC-like cells were most similar to wild-type OHCs at ~P1. (A)** Drawing  
1041 illustrating manual picking of Tdtomato+ cells (of different cell types from three  
1042 different models) and single-cell RNA-Seq. **(B-C””**) Triple labeling for Myo7a, Prestin,  
1043 and Tdtomato in cochlear samples from control (B) and *Fgfr3-Atoh1-Ikzf2-DTR* (C)  
1044 mice at P60. Arrows: Tdtomato+/Myo7a+/Prestin+ OHC-like cell; arrowheads:  
1045 Tdtomato+/Myo7a-/Prestin- cell, defined as SC that failed to become an HC; asterisks:  
1046 nascent HC that was Tdtomato+/Myo7a+/Prestin- (inset in C-C””). **(D)** UMAP analysis  
1047 of five different cell types together. All cells were Tdtomato+, but were picked from  
1048 three different models. The 42 Tdtomato+ cells within the light-blue dotted lines were  
1049 from *Fgfr3-Atoh1-Ikzf2-DTR* mice at P60; these cells were divided into three sub-  
1050 clusters. **(E)** Pearson correlation-coefficient analysis of the same five cell types as in  
1051 (D). **(F-G)** UMAP analysis of all cells in (D-E), except for SCs that failed to become  
1052 HCs, together with wild-type OHCs at E16, P1, and P7 (E16\_WT OHCs, P1\_WT OHCs,  
1053 and P7\_WT OHCs; detailed information in Supplemental Figure 8).

1054

1055

1056 **SUPPLEMENTAL FIGURE LEGENDS**

1057 **Figure 1-figure supplement 1. Generation of *Rosa26-CAG-LSL-Ikzf2/+* knockin**  
1058 **mouse model by using CRISPR/Cas9 method. (A)** Wild-type *Rosa26* allele. **(B)**  
1059 Illustration of target vector. *Ikzf2* was tagged with 3× HA fragments at its C-terminus,  
1060 and this was followed by T2A-Tdtomato; *Ikzf2* and Tdtomato were transcribed and  
1061 translated together as a fusion protein, but then cleaved into *Ikzf2* and Tdtomato,  
1062 respectively, through the 2A approach. **(C)** Illustration of *Rosa26* after correct gene  
1063 targeting. **(D-E)** Southern blotting assay of internal probe (D) and 3'-end probe (E). **(F)**  
1064 Genotyping PCR of tail DNA extracted from heterozygous (KI/WT) and wild-type  
1065 (WT/WT) mice. WT mice showed a single 609-bp band, whereas heterozygous mice  
1066 showed 609- and 340-bp bands.

1067

1068 **Figure 1-figure supplement 2. Prestin was expressed in IHCs with ectopic *Ikzf2*.**  
1069 **(A)** Same as illustration in Figure 1A. **(B-C””)** Triple labeling for Prestin, Tdtomato,  
1070 and vGlut3 in both control *Atoh1-CreER*<sup>+</sup> mice (B-B””)  
1071 and experimental *Atoh1-CreER*<sup>+</sup>; *Rosa26-CAG-LSL-Ikzf2/+* mice (C-C””)  
1072 at P42; these low-magnification images compliment the images in Figure 1. Prestin was expressed in vGlut3+ IHCs of  
1073 experimental but not control mice, and Prestin expression again completely overlapped  
1074 with that of Tdtomato. Scale bar: 200  $\mu$ m.

1075

1076 **Figure 3-figure supplement 1. Generation of *Prestin-DTR/+* knockin mouse model.**  
1077 **(A)** Wild-type *Prestin* (*Slc26a5*) allele. **(B)** Illustration of target vector. P2A-DTR

1078 (diphtheria toxin receptor) was inserted immediately before the stop codon *TAA*. DTR  
1079 expression was completely controlled by *Prestin* promoter and enhancer. **(C)**  
1080 Illustration of *Prestin* allele after correct gene targeting. **(D-E)** Southern blotting assay  
1081 of internal DTR probe (D) and 5'-end probe (E). **(F)** Genotyping PCR of tail DNA  
1082 extracted from heterozygous (KI/WT) and wild-type (WT/WT) mice. WT mice  
1083 displayed a single 269-bp band, whereas heterozygous mice showed 195- and 269-bp  
1084 bands.

1085

1086 **Figure 4-figure supplement 1. Nascent HCs emerged at P42 and OHC-like cells at**  
1087 **P46. (A-C'')** Triple labeling for early HC marker Myo6, Tdtomato, and Prestin in  
1088 control mice at P42 (A-A'') and in experimental mice at P42 (B-B'') and P46 (C-C'').  
1089 Arrowheads: nascent HCs that were Tdtomato+/Myo6+/Prestin-; arrows: OHC-like  
1090 cells that were Tdtomato+/Myo6+/Prestin+; asterisks: debris of dying endogenous  
1091 OHCs. **(D)** Quantification of new HCs at different cochlear turns in *Fgfr3-Atoh1-Ikzf2-*  
1092 *DTR* mice at P42 and P46. More new HCs tended to be present at P46 than P42,  
1093 although no statistical difference was calculated due to large variations in the numbers;  
1094 mean numbers of nascent HCs are shown. **(E)** Quantification of new HCs (nascent and  
1095 OHC-like cells together) and OHC-like cells at the same turn in *Fgfr3-Atoh1-Ikzf2-*  
1096 *DTR* mice at P46; mean numbers of new HCs and OHC-like cells were shown. Scale  
1097 bar: 20  $\mu$ m.

1098

1099 **Figure 6-figure supplement 1. Transcriptomic comparison between adult wild-**

1100 **type OHCs and SCs.** We manually picked 17 wild-type OHCs at P30 (P30\_WT OHCs)  
1101 and 16 SCs at P60 (P60\_WT SCs) and performed single-cell RNA-Seq by using the  
1102 smartseq approach. **(A)** Examples of top enriched genes in P30\_WT OHCs and  
1103 P60\_WT SCs; the complete gene list appears in Supplemental File 1. Early pan-HC-  
1104 specific genes such as *Myo7a* (red arrow) and OHC-specific genes such as *Ocm*, *Lbh*,  
1105 *Prestin* (*Slc26a5*), and *Ikzf2* were incorporated with the genes enriched in P30\_WT  
1106 OHCs. Similarly, SC markers such as *Sox2*, *Sox10*, *Bace2*, and *Ceacam16* were  
1107 included among the genes enriched in P60\_WT SCs. As expected, the IHC-specific  
1108 gene *Slc7a14* was included with neither OHC- nor SC-enriched genes. **(B-C)** Gene  
1109 Ontology (GO) enrichment analysis of genes enriched in OHCs (B, green) and SCs (C,  
1110 gray). The gene lists of each GO category appeared in Supplemental Files 2 and 3.

1111

1112 **Figure 6-figure supplement 2. Hundreds OHC and SC genes were upregulated and**  
1113 **downregulated, respectively, in OHC-like cells.** Single-cell RNA-Seq was applied to  
1114 42 Tdtomato+ cells from *Fgfr3-Atoh1-Ikzf2-DTR* mice at P60; the cells were  
1115 categorized as OHC-like cells, nascent HCs, and SCs that failed to become HCs. We  
1116 compared the transcriptomic profiles between OHC-like cells and P60\_WT SCs. **(A)**  
1117 Relative to P60\_WT SCs, top significantly upregulated or downregulated genes in  
1118 OHC-like cells were sorted first and only those that overlapped with genes enriched in  
1119 P30\_WT OHCs or P60\_WT SCs were selected and presented as examples. Here,  
1120 nascent HCs and SCs that failed to become HCs were included as references only. The  
1121 complete gene lists were presented in Supplemental File 4. **(B)** GO analysis of all genes

1122 (without using overlap with genes enriched in P30\_WT OHCs) that were significantly  
1123 upregulated in OHC-like cells. The gene lists of each GO category appeared in  
1124 Supplemental File 5.

1125

1126 **Figure 6-figure supplement 3. Heterogeneous expression of OHC genes in OHC-**  
1127 **like cells.** Cochlear samples (P60) from control *Prestin-DTR/+* mice not treated with  
1128 DT (A, C, E, G) and experimental *Fgfr3-Atoh1-Ikzf2-DTR* mice treated with DT (B-  
1129 B'', D-D'', F-F'', H-H'') were triple labeled for Tdtomato (red), Prestin (white in  
1130 images), and a third different marker (antibody staining, shown in green): Insm1 (A-  
1131 B''), Rbm24 (C-D''), Pvalb (E-F''), or Calb1 (G-H''). Asterisks in (B-B''): cell that  
1132 was Tdtomato+/Insm1+ but did not yet express Prestin; arrows in (B-B''): cell that was  
1133 Tdtomato+/Insm1+/Prestin+. Inset in (A): cochlear cryosection sample at E16.5 stained  
1134 for Myo6 and Insm1. Insm1 expression was detected in OHCs but not in IHCs, which  
1135 was presented as another control to confirm the specificity of the Insm1 antibody.  
1136 Arrows in (D-D'', F-F'', H-H''): triple-positive cells; arrowheads in (D-D'', F-F'',  
1137 H-H''): cells that were Tdtomato+/Prestin+ but did not express Rbm24 (D-D''), Pvalb  
1138 (F-F''), or Calb1 (H-H''). Prestin in (B'', D'', F'', H'') was shown at a higher gain  
1139 than in (A, C, E, G) to enhance signal visualization; the Prestin level in OHC-like cells  
1140 was considerably lower than that in wild-type OHCs. Scale bar: 20  $\mu$ m.

1141

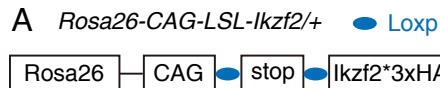
1142 **Figure 6-figure supplement 4. Trajectory analysis of wild type differentiating**  
1143 **OHCs. (A)** UMAP analysis of wild-type OHCs at E16, P1, and P7. OHCs of the same

1144 age formed their own main cluster. **(B-B')** Trajectory analysis of OHCs at three  
1145 different ages by using Monocle. Cells within dotted lines were selected for further  
1146 analysis as described in Figure 6.

1147

1148 **Figure 6-figure supplement 5. Transcriptomic difference between OHC-like cells**  
1149 **and mature OHCs. (A)** Examples of top DEGs between OHC-like cells and wild-type  
1150 OHCs at P30 (P30\_WT OHCs). Nascent HCs and SCs that failed to become HCs were  
1151 included as references only. *Atoh1*, *Insm1*, and *Hes6*, which are transiently expressed  
1152 in OHCs, were expressed at a higher level in OHC-like cells than in P30\_WT OHCs.  
1153 *Id2*, *Id3*, and *Fgfr3* are normally expressed in SCs but were not significantly decreased  
1154 in OHC-like cells; these genes were indicated by purple arrows. Conversely, *Ocm*,  
1155 *Prestin* (*Slc26a5*), *Lmo7*, and *Tmc1* showed increased expression in P30\_WT OHCs,  
1156 and these genes encode functional proteins in mature OHCs (green arrows). The  
1157 complete gene lists were presented in Supplemental File 6. **(B-C)** GO analysis of genes  
1158 upregulated in OHC-like cells (B) or P30\_WT OHCs (C). The gene lists of each GO  
1159 category were included in Supplemental Files 7 and 8, respectively.

Fig. 1



Prestin

## Tdtomato

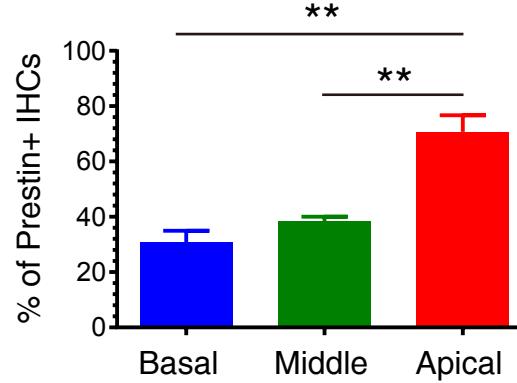
### vGlut3

## Merge + Hoechst

Atoh1-CreER<sup>+</sup>  
(CTRL)

B

Atoh1-CreER+;  
Rosa26-CAG-LSL-Lyzf2/+



HA (Ikzf2) + Tdtomato

Atoh1-CreER+  
(CTRL)

Atoh1-CreER+;  
Rosa26-CAG-LSL-Ikzf2/+

A fluorescence micrograph showing a layer of cells. The nuclei are stained blue, and the cytoplasm is stained red. The label 'P4' is in the top right corner, and 'E' is in the top left corner.

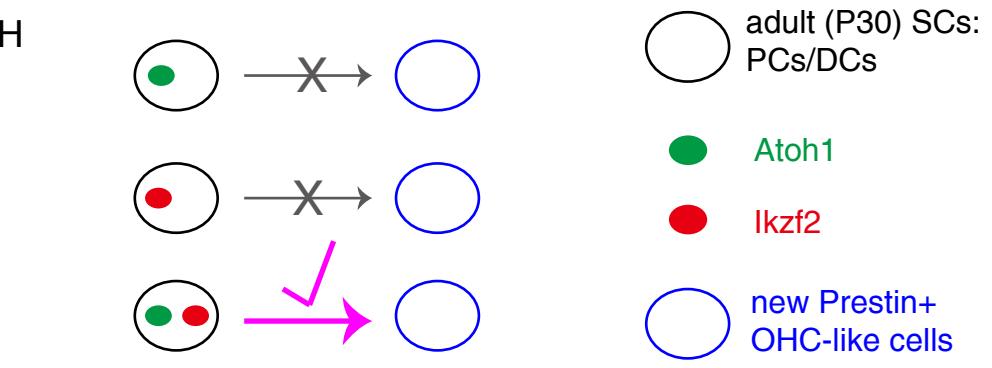
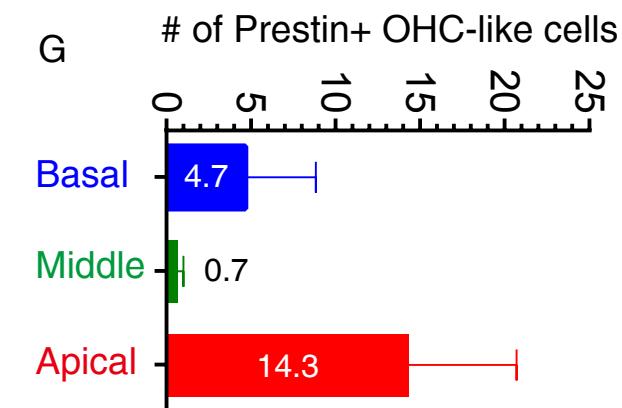
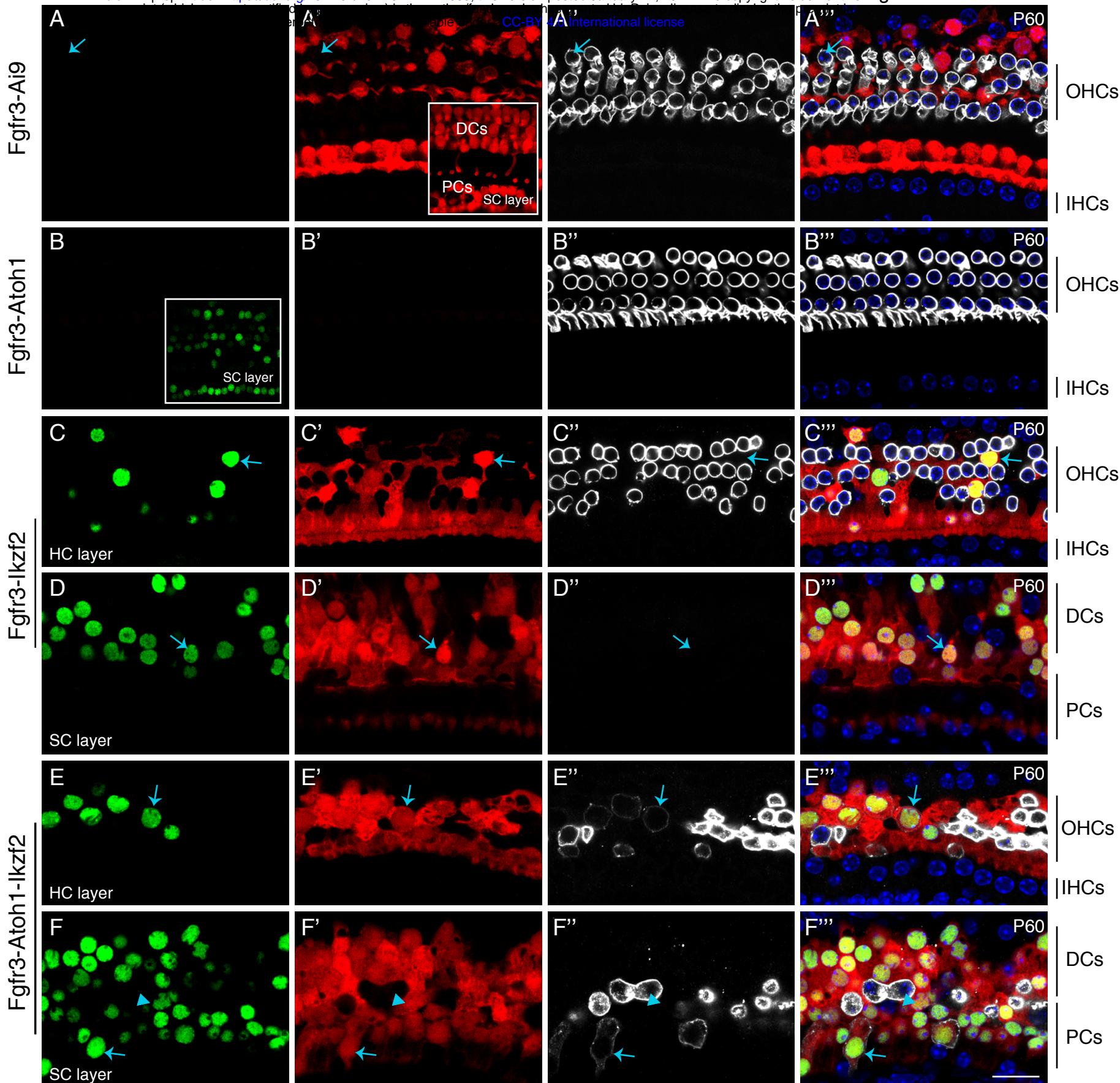


Fig. 3

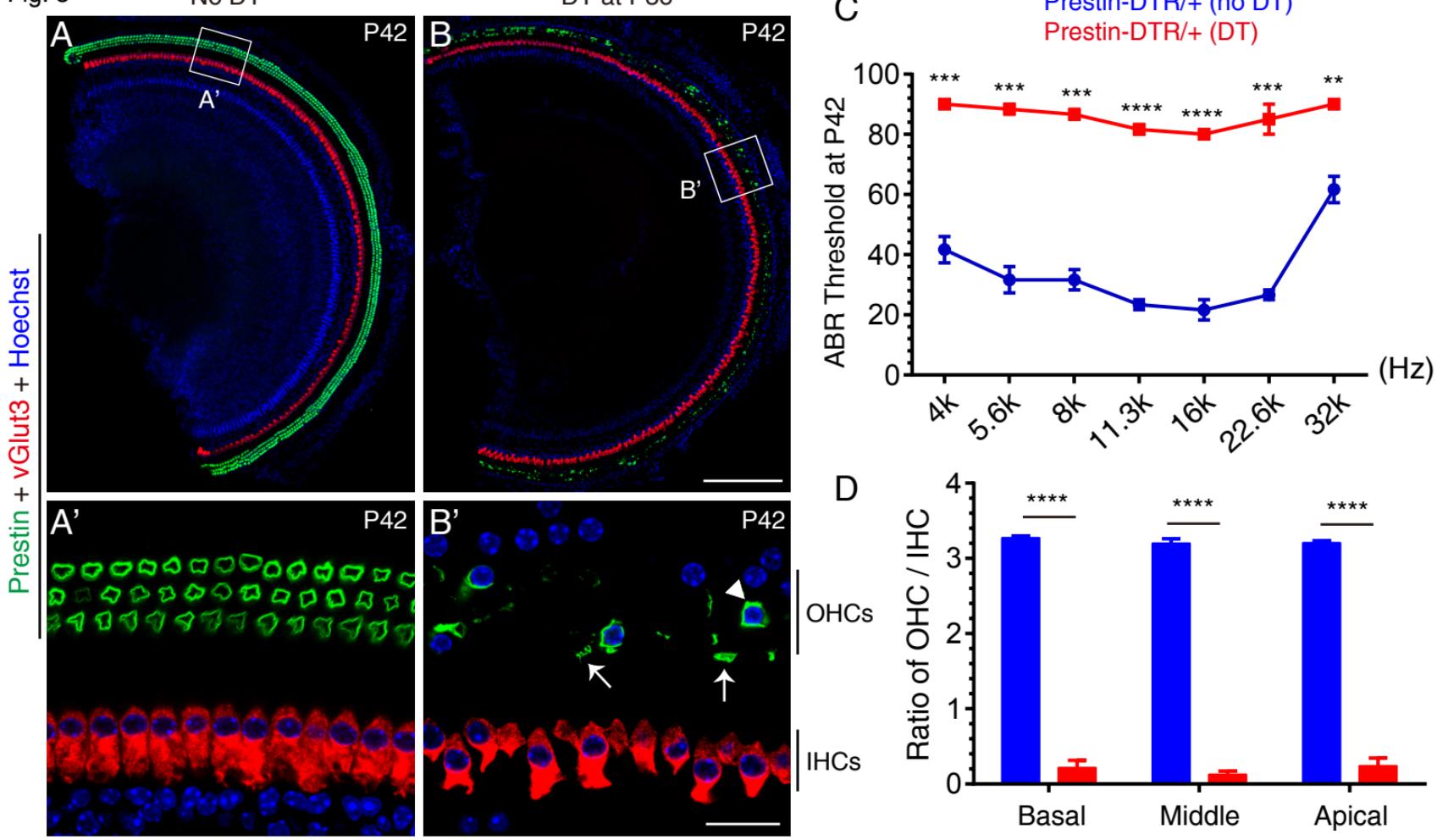


Fig. 4

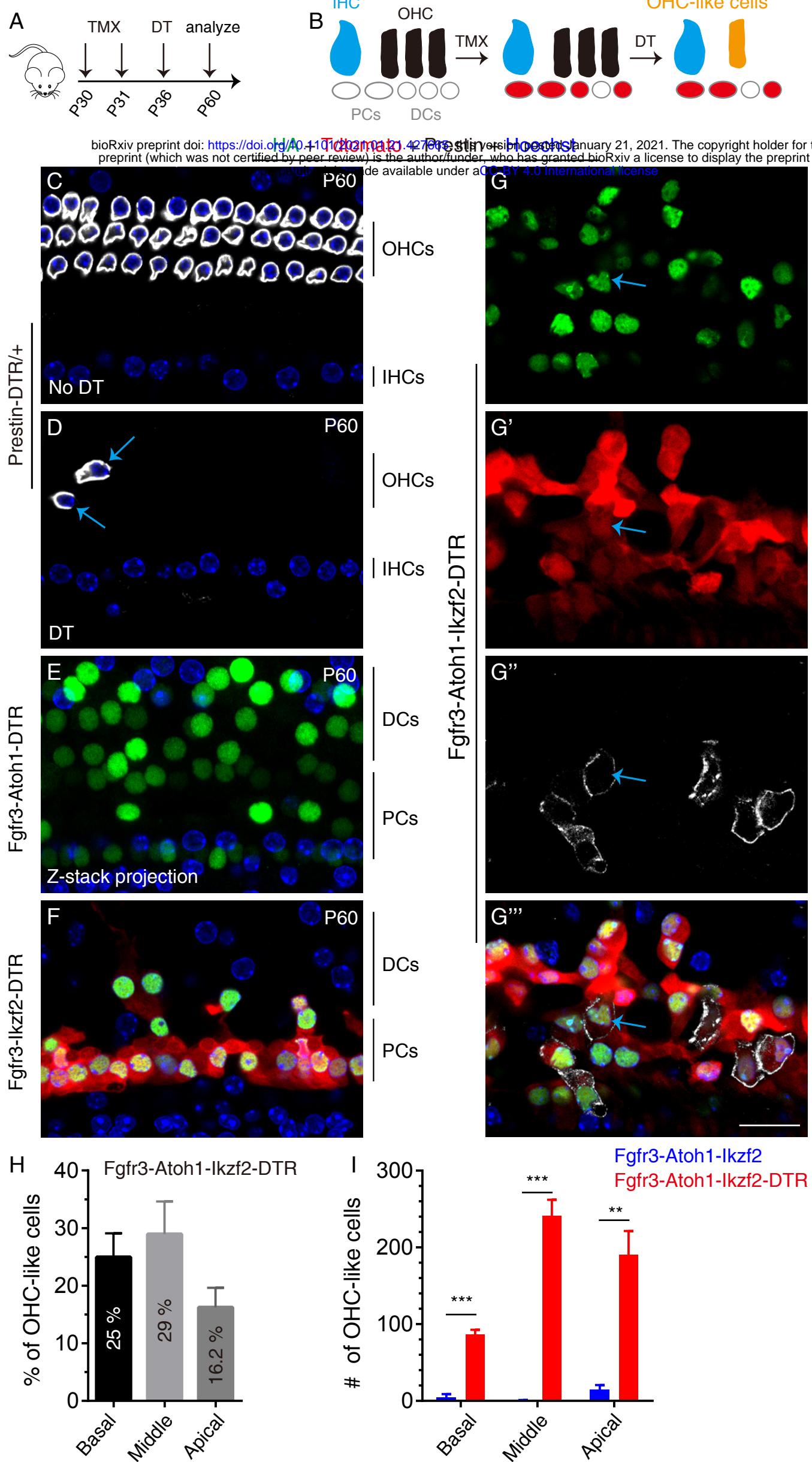
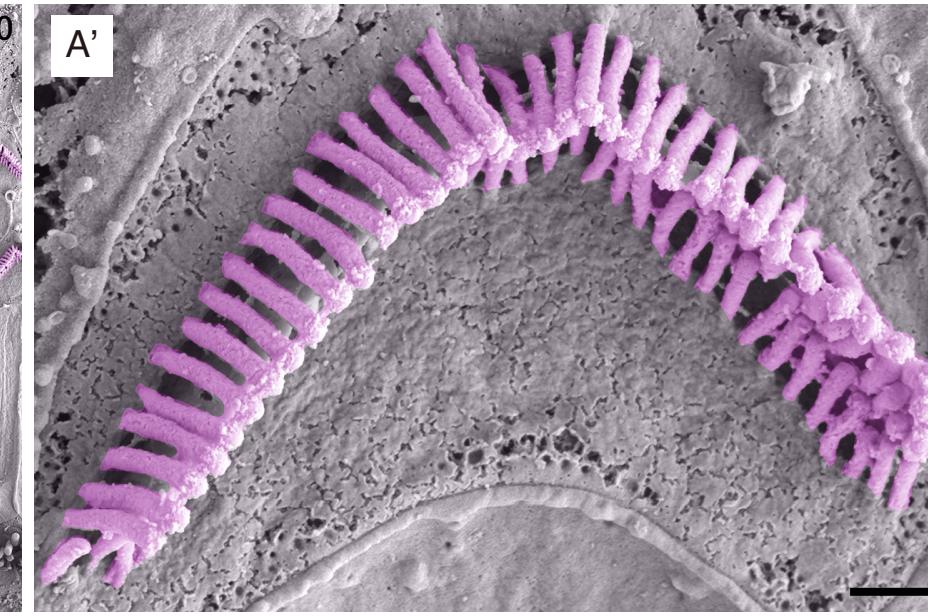
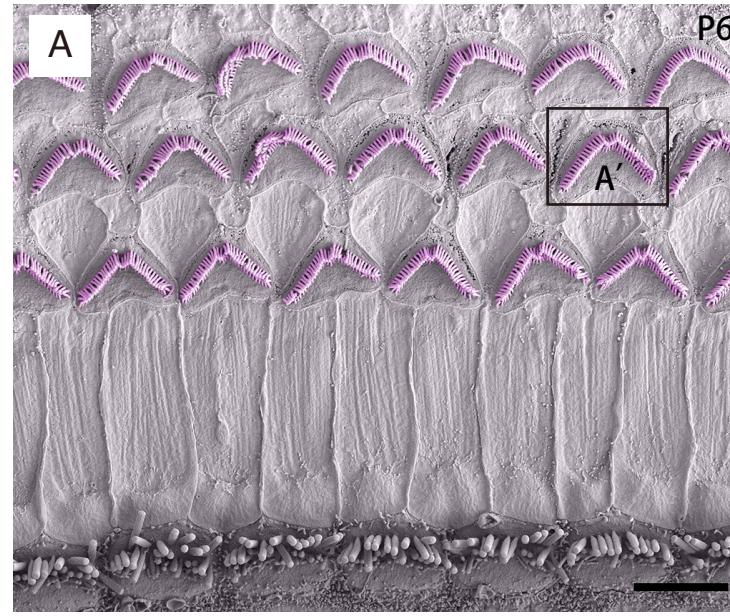
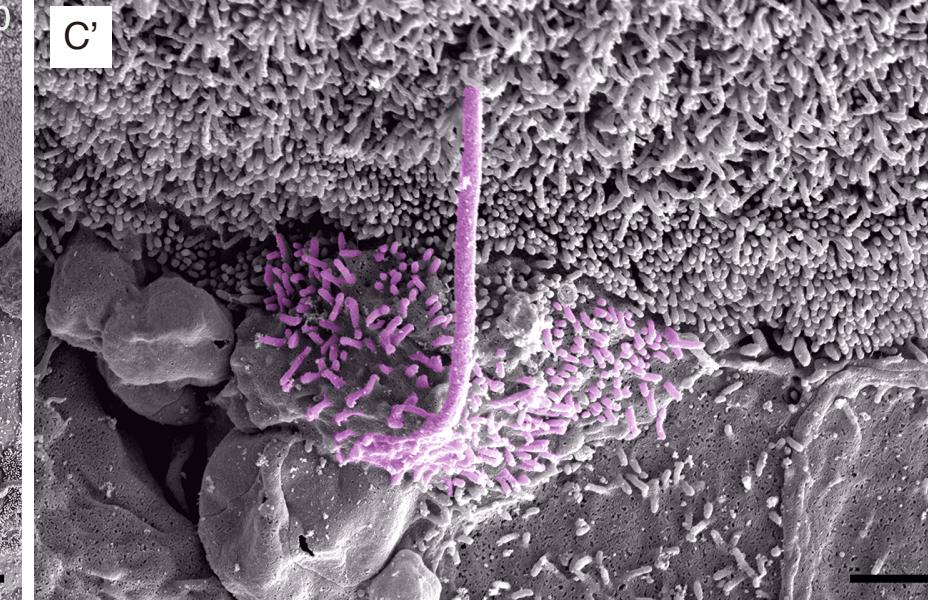
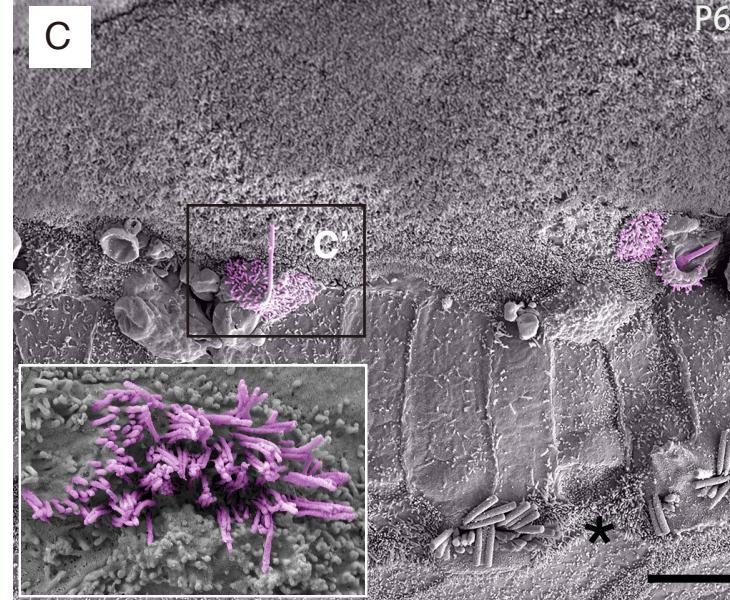


Fig. 5

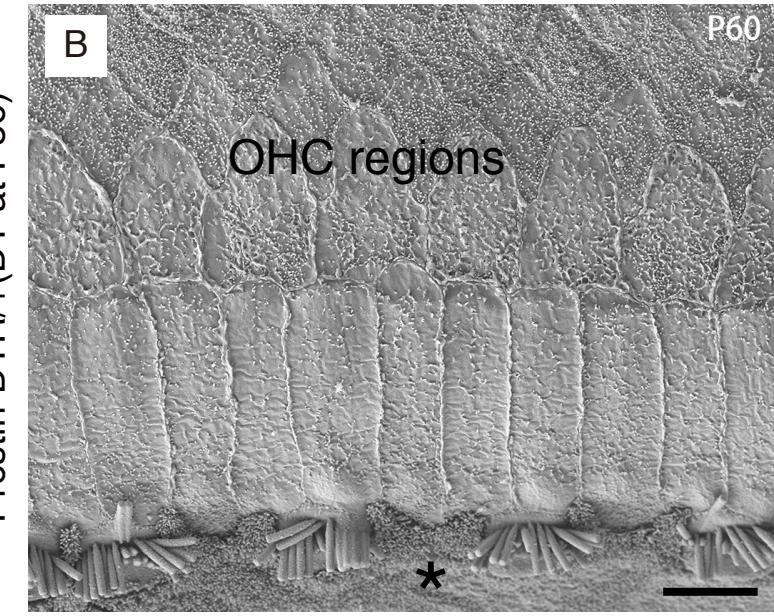
Egrfr3-Atoh1-|kzf2-DTR Prestin-DTR/±(NO DT)



P6



Prestin-DTB/+(DT at P36)



1

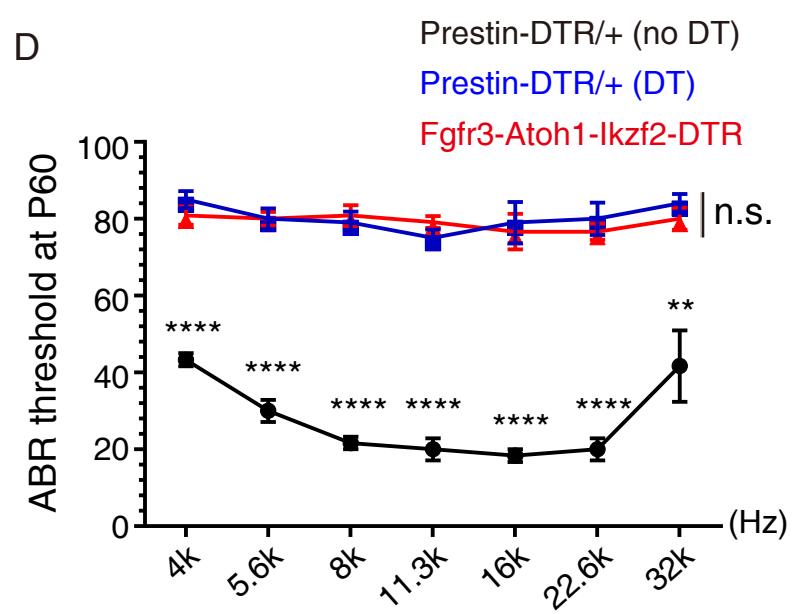


Fig. 6

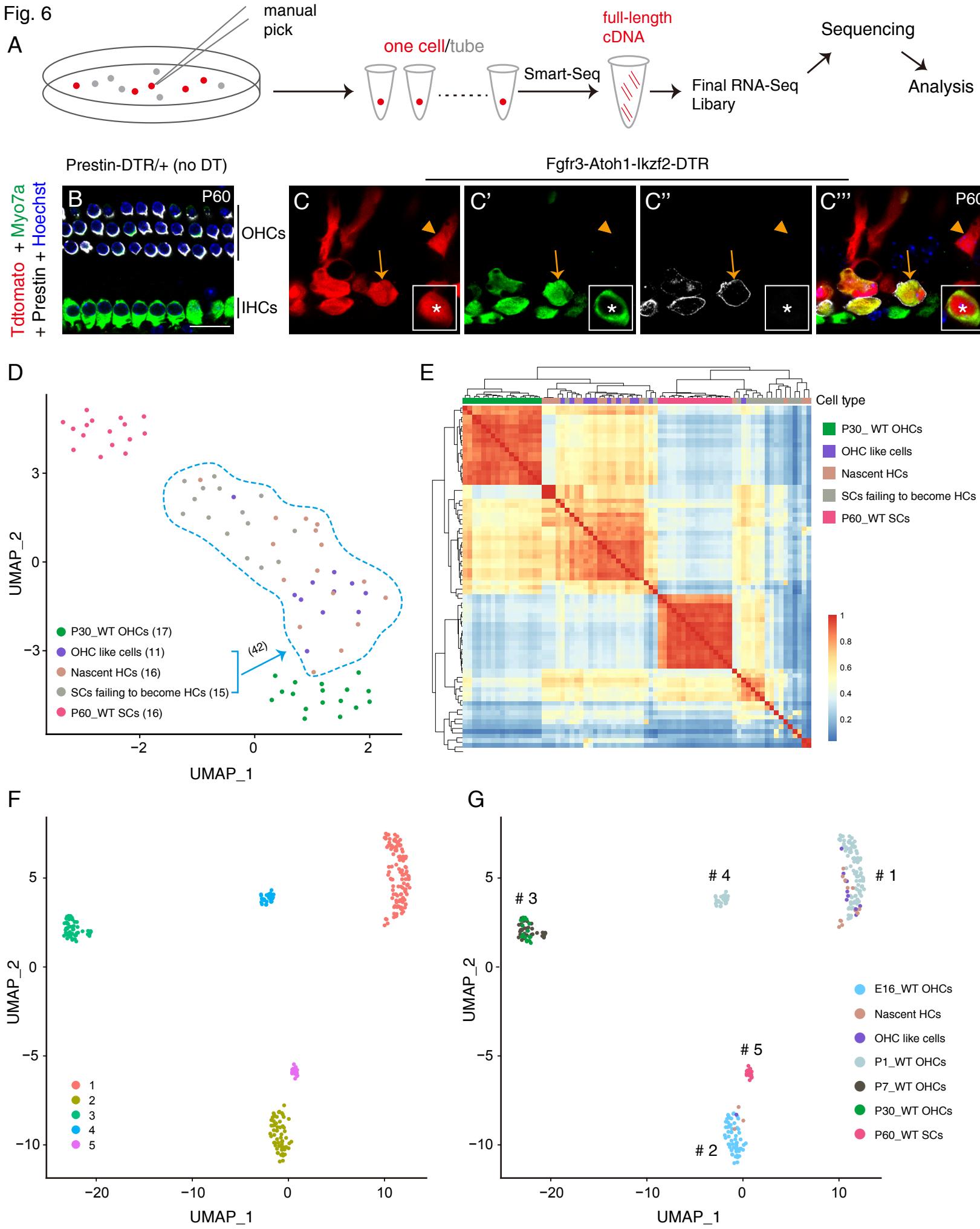


Figure 1-figure supplement 1

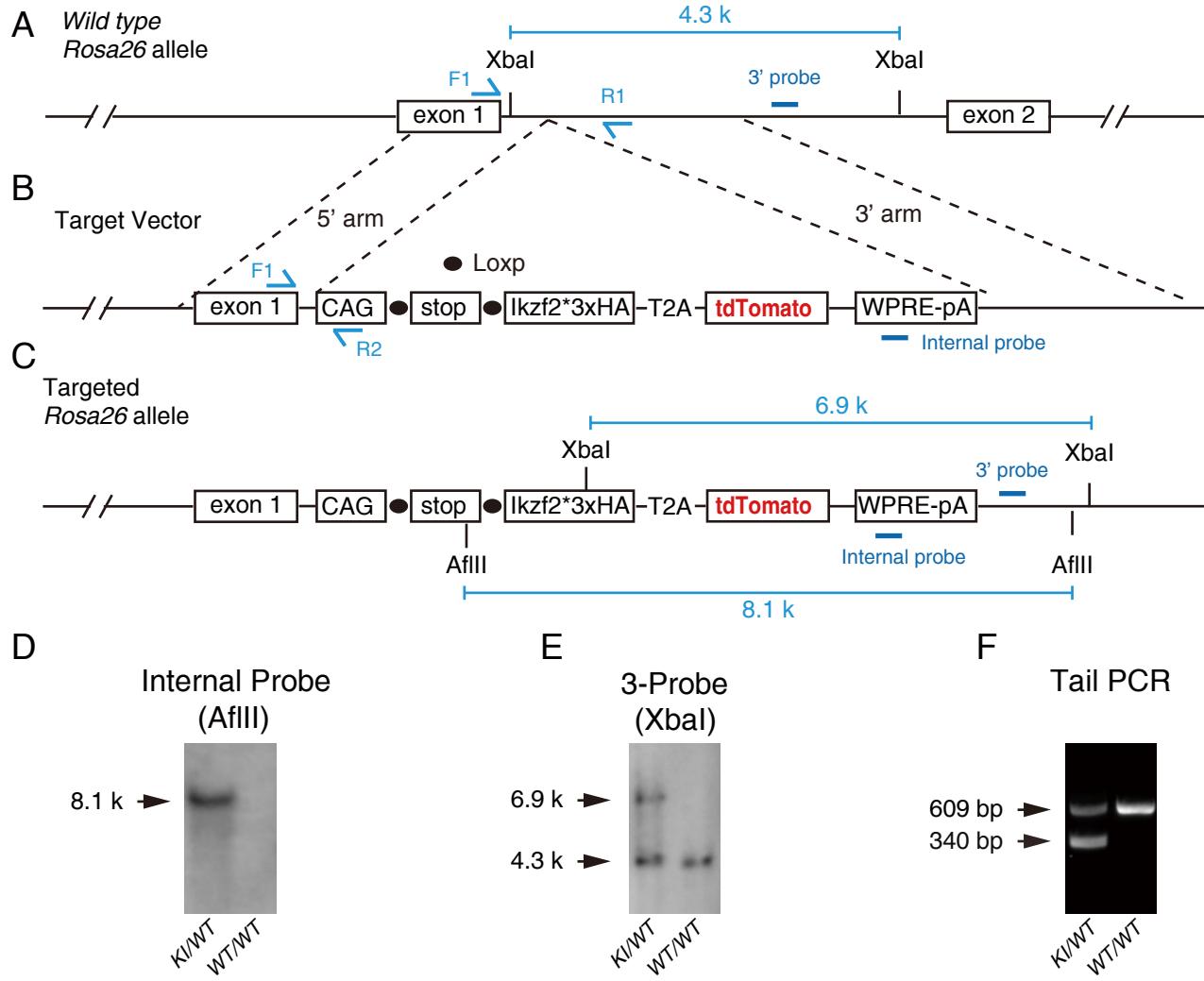
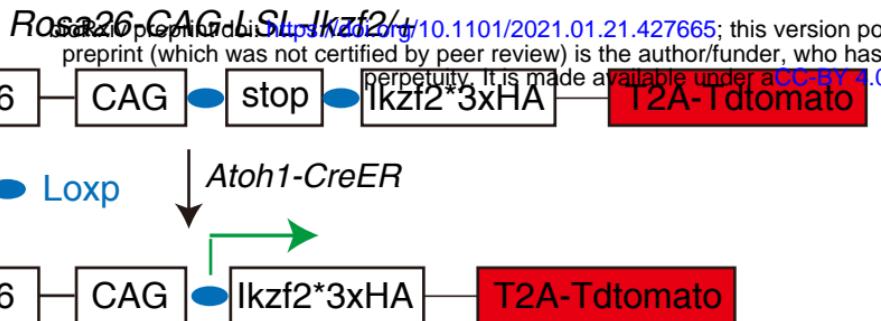


Figure 1-figure supplement 2

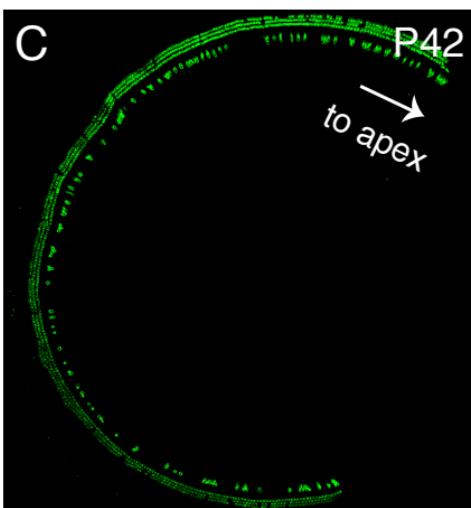
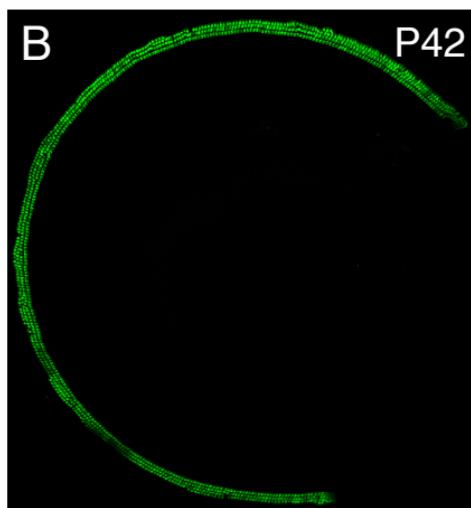
A



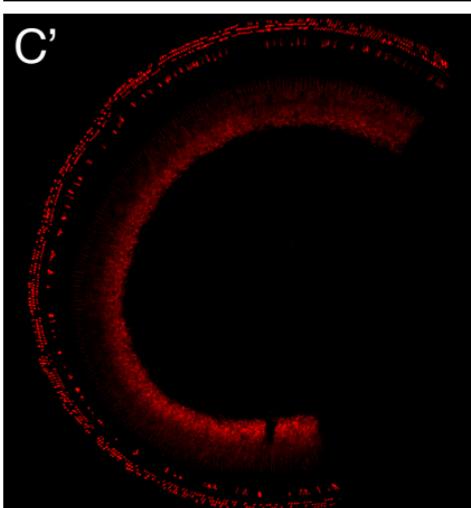
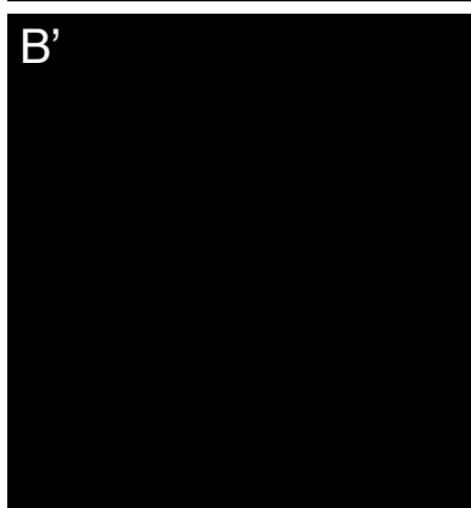
Atoh1-CreER+  
(CTRL)

Atoh1-CreER+;  
Rosa26-CAG-LSL-lkzf2+/+

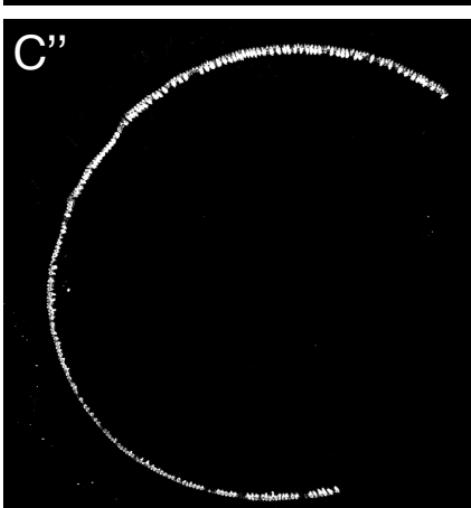
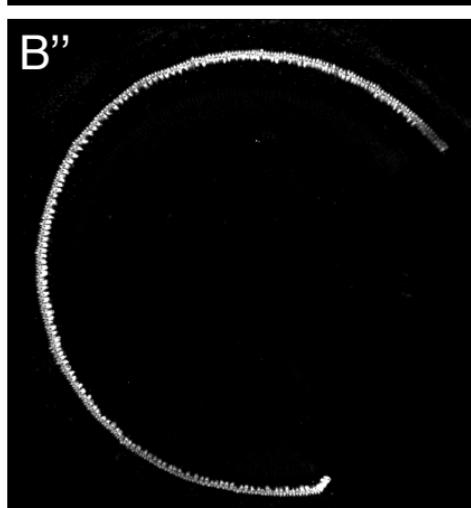
Prestin



Tdtomato



vGlut3



Merge + Hoechst

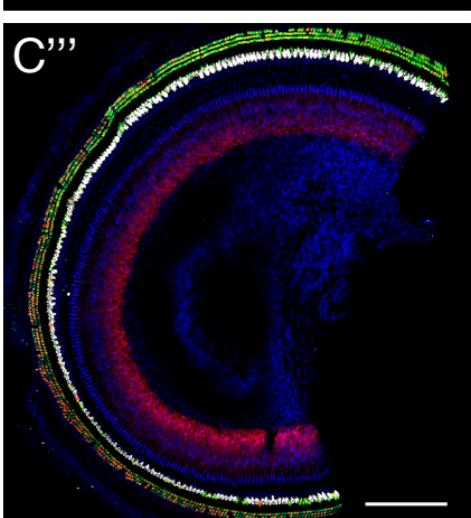
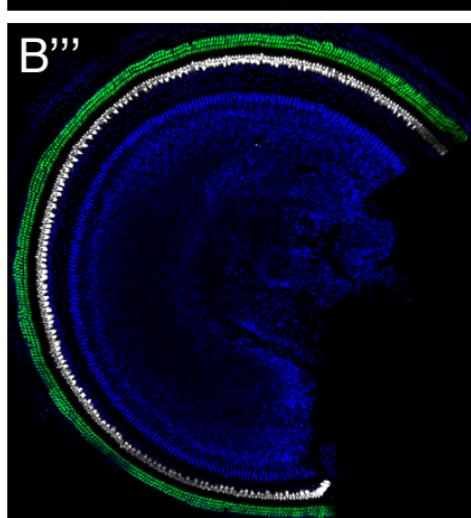
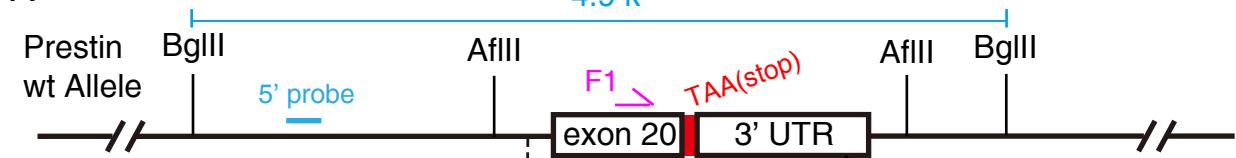


Figure 3-figure supplement 1

A



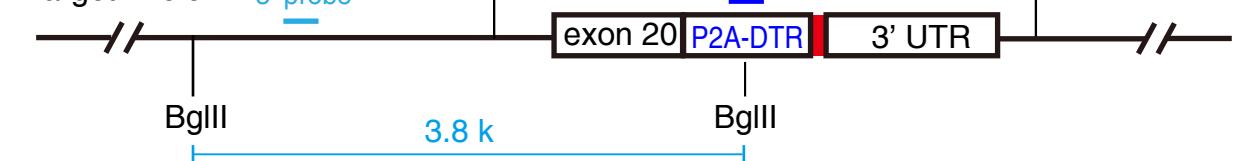
B

Target Vector



C

Target Allele



D

DTR Probe  
(AfIII)

3.3 k →



KI/WT  
WT/WT

E

5' Probe  
(BgIII)

4.9 k →  
3.8 k →

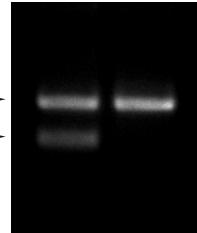


KI/WT  
WT/WT

F

Tail PCR

269 bp →  
195 bp →



KI/WT  
WT/WT

Figure 4-figure supplement 1

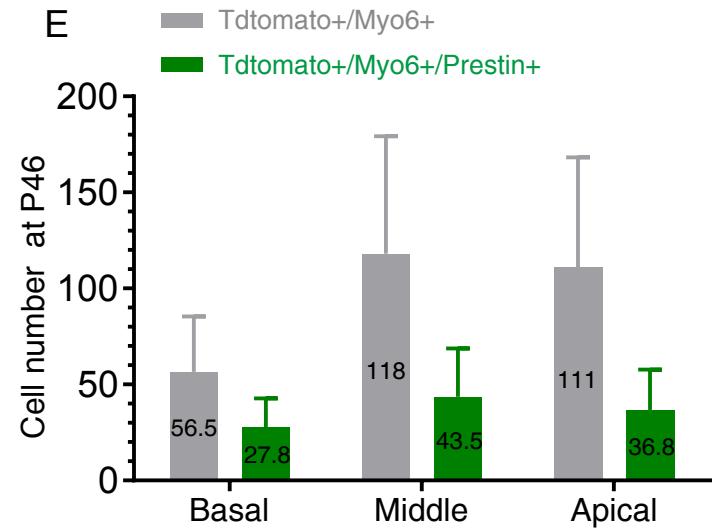
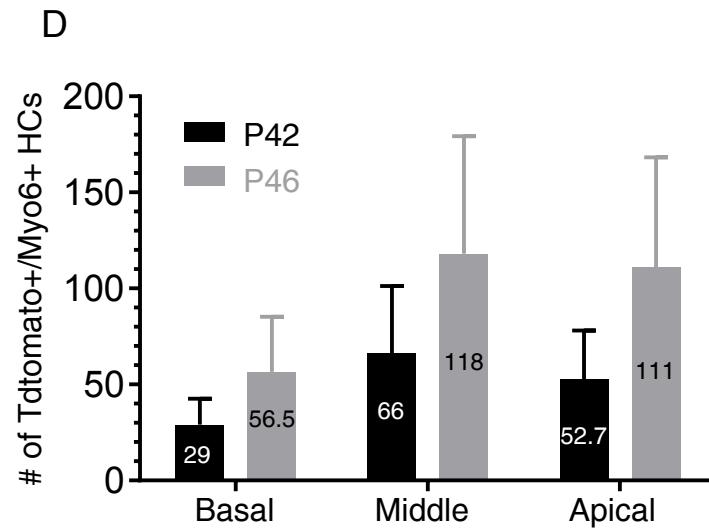
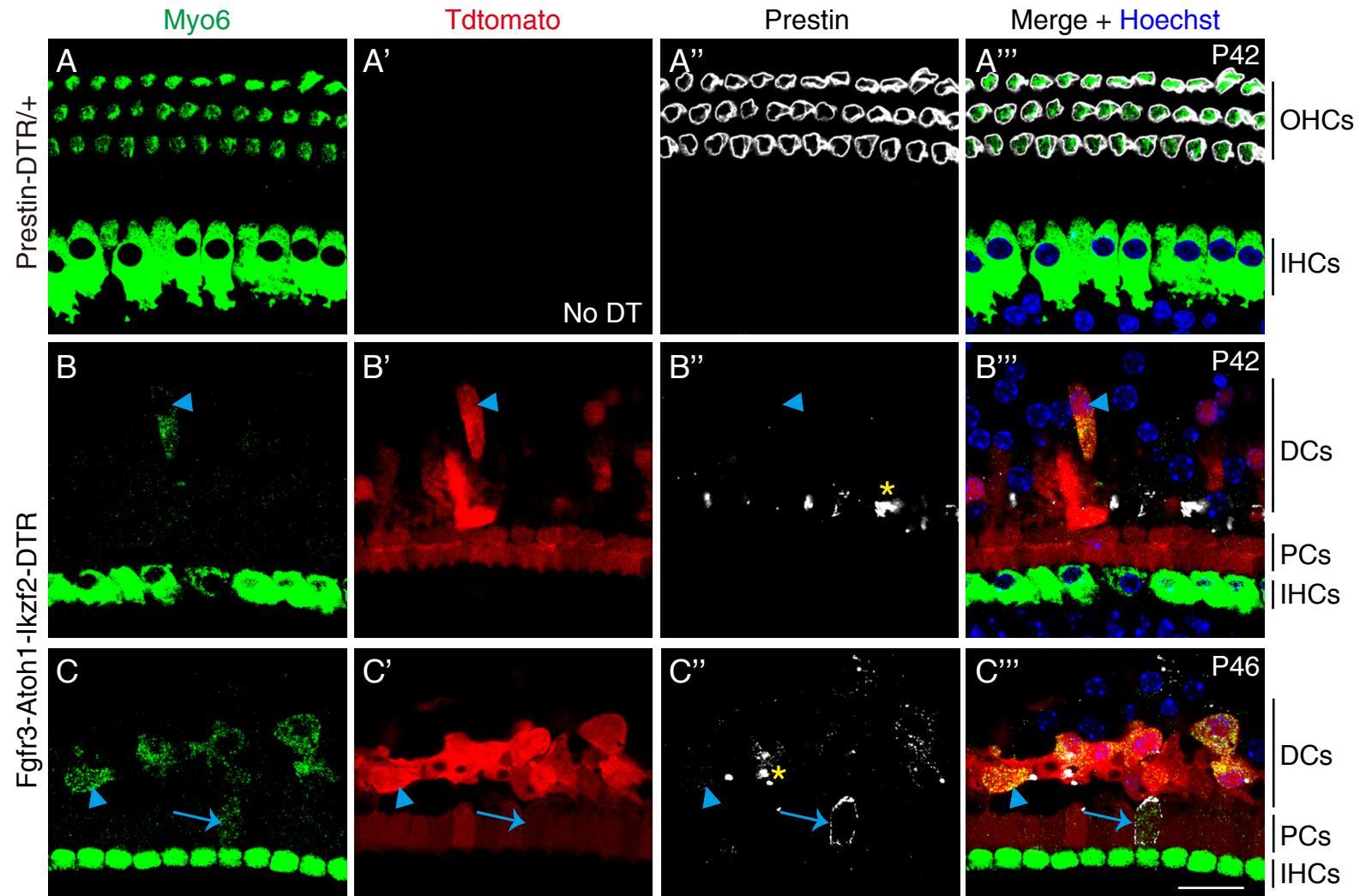
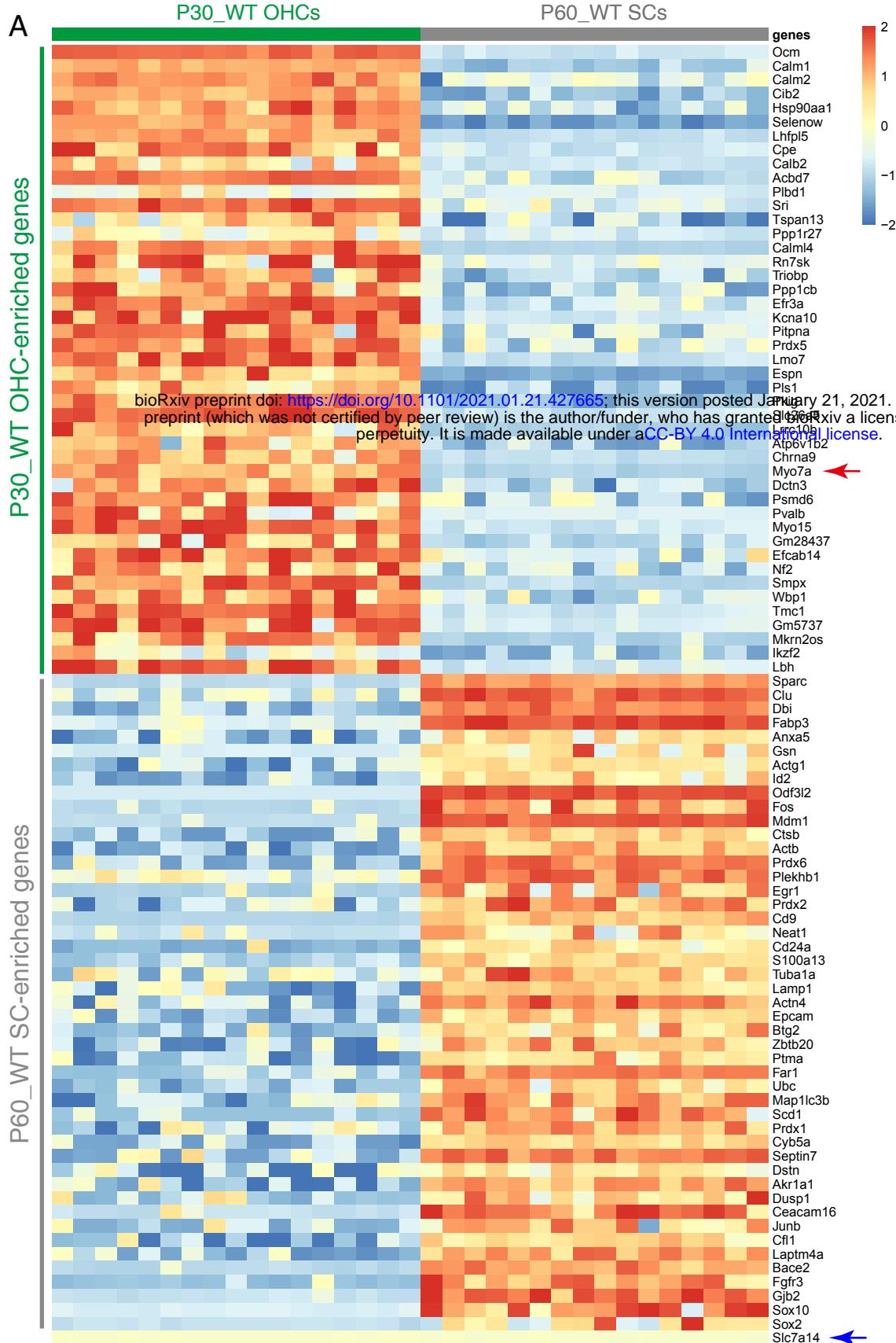
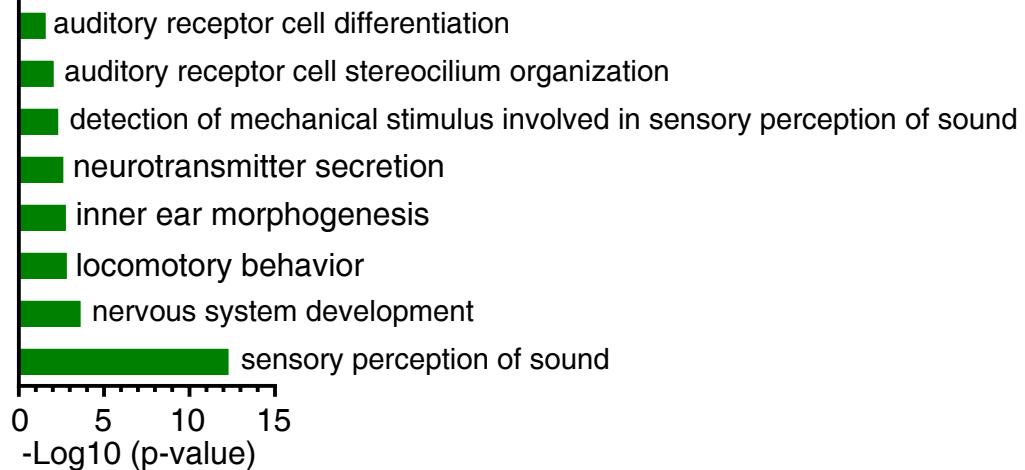


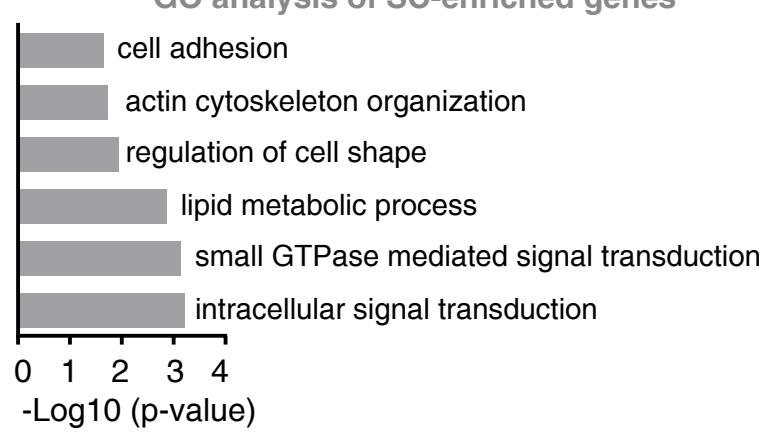
Figure 6-figure supplement 1



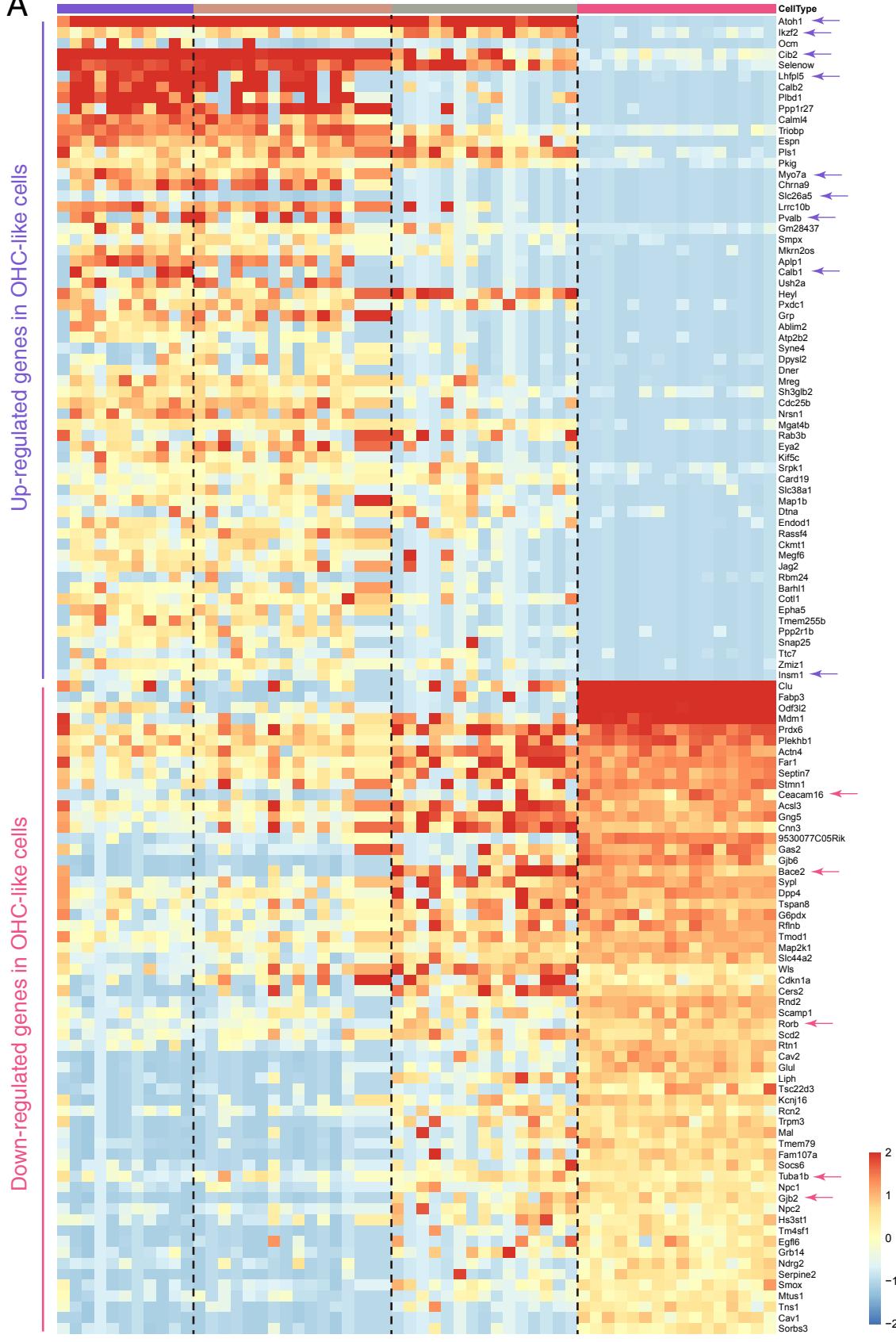
**B GO analysis of OHC-enriched genes**



**C GO analysis of SC-enriched genes**

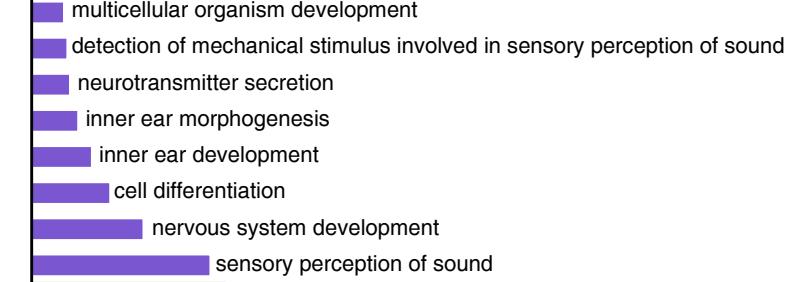


A



B

GO analysis of up-regulated genes in OHC-like cells (compared to P60\_WT SCs)



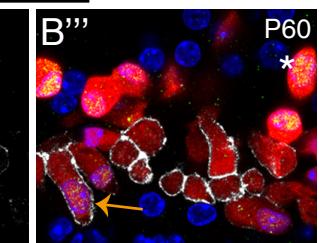
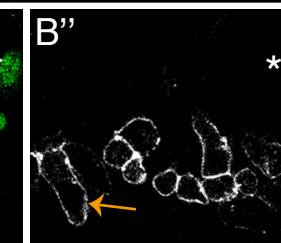
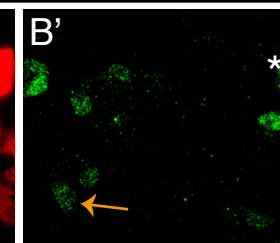
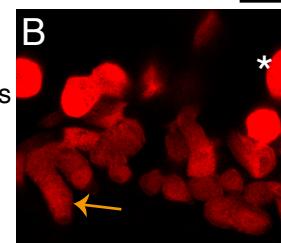
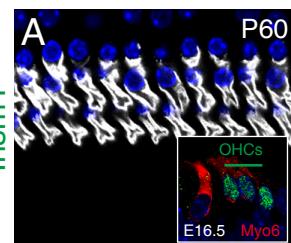
0 2 4 6 8  
-Log10 (p-value)

Figure 6-figure supplement 3

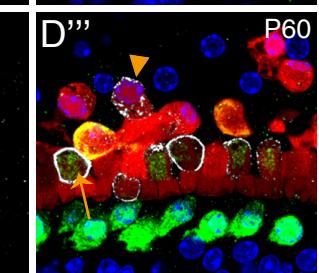
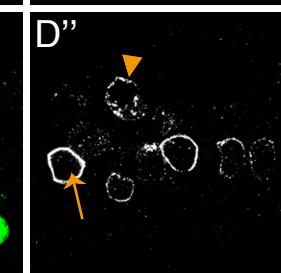
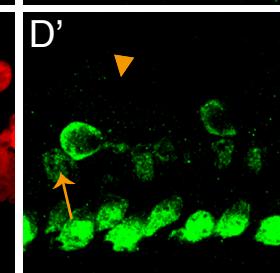
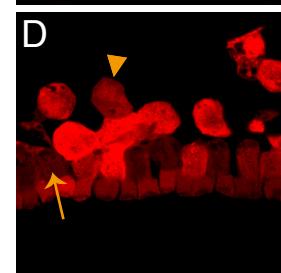
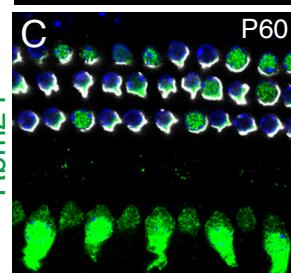
Prestin-DTR/+ (no DT)

Fgfr3-Atoh1-Ikzf2-DTR

Tdtomato + Prestin + Hoechst



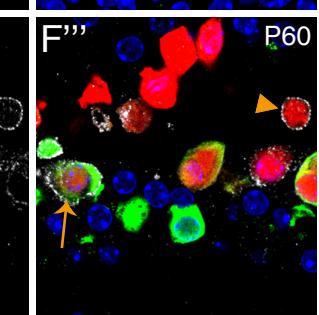
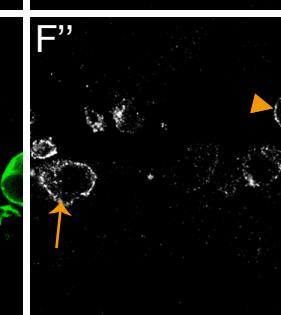
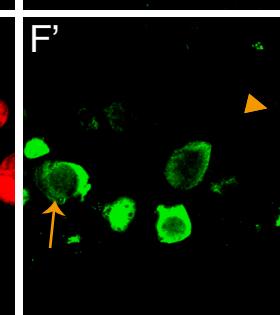
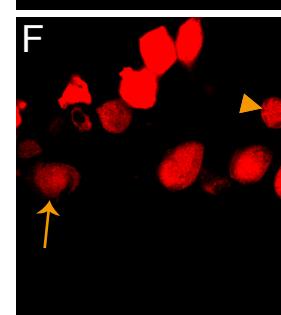
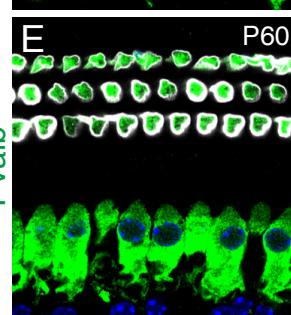
InsM1



Rbm24



Pvalb



Calb1

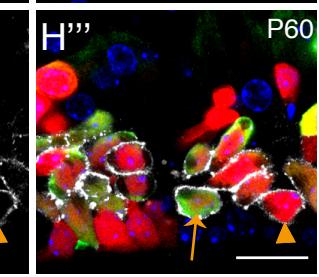
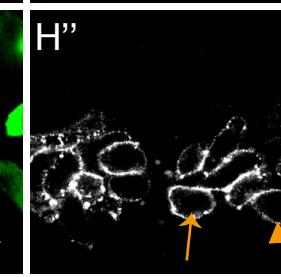
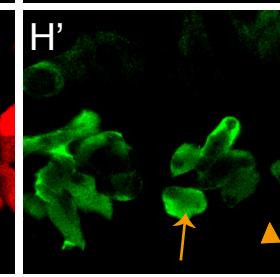
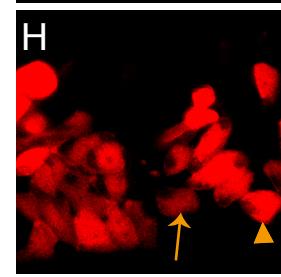
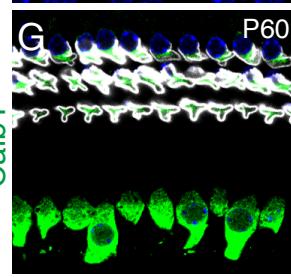


Figure 6-figure supplement 4

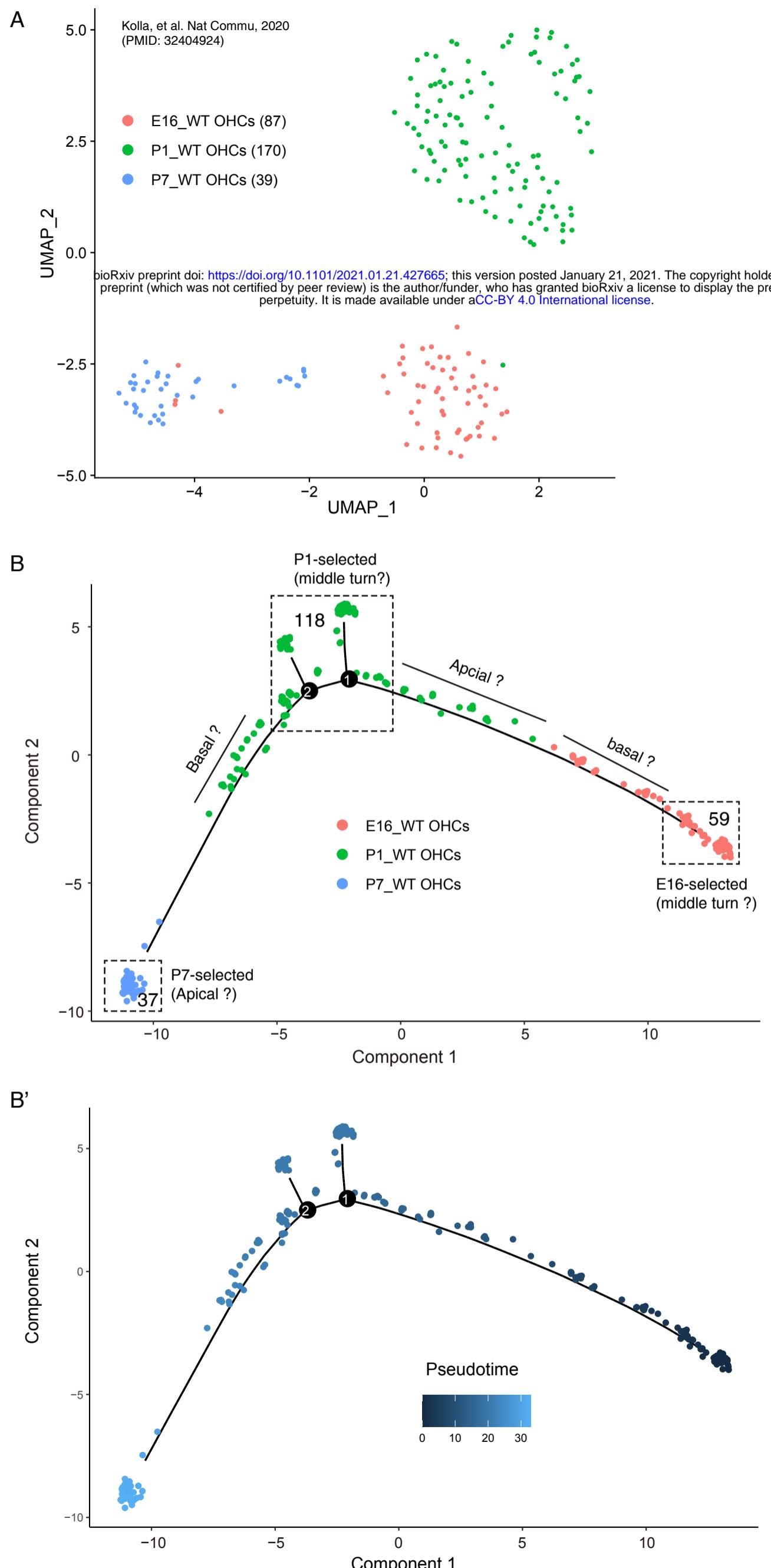
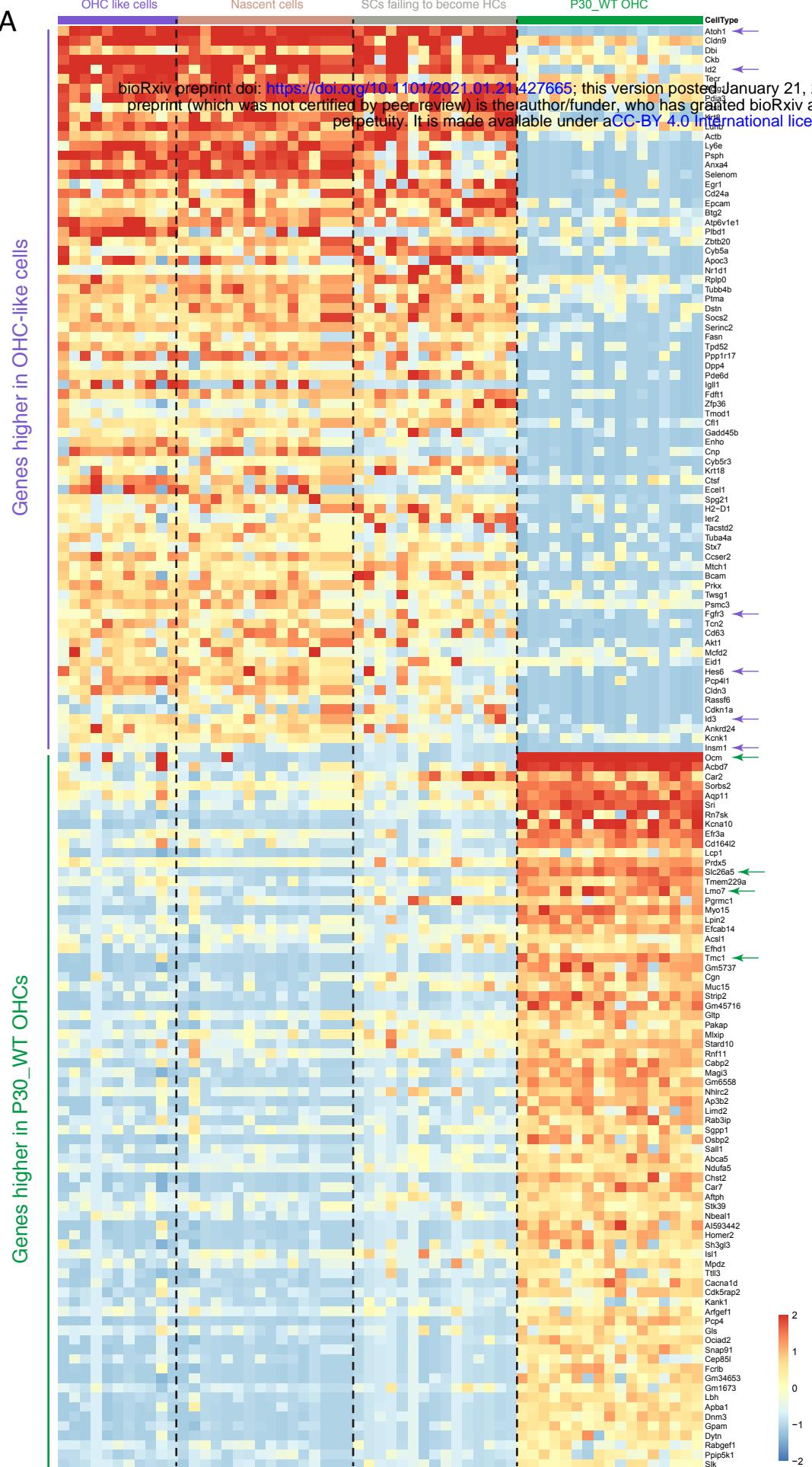
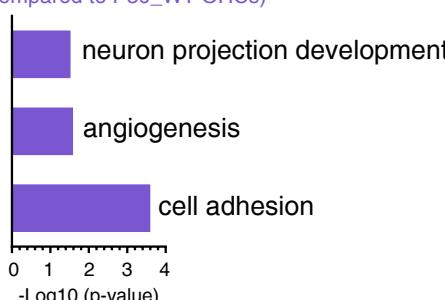


Figure 6-figure supplement 5

A



B GO analysis of genes enriched in OHC-like cells (compared to P30\_WT OHCS)



C GO analysis of genes enriched in P30\_WT OHCS (compared to OHC-like cells)

