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3 **TITLE:** Activin signaling informs the graded pattern of terminal mitosis and hair cell

4 differentiation in the mammalian cochlea

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6 **SHORT TITLE:** Activin signaling instructs auditory hair cell differentiation

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26 **ABSTRACT**

27 The mammalian auditory sensory epithelium has one of the most stereotyped cellular
28 patterns known in vertebrates. Mechano-sensory hair cells are arranged in precise rows, with
29 one row of inner and three rows of outer hair cells spanning the length of the spiral-shaped
30 sensory epithelium. Aiding such precise cellular patterning, differentiation of the auditory
31 sensory epithelium is precisely timed and follows a steep longitudinal gradient. The molecular
32 signals that promote auditory sensory differentiation and instruct its graded pattern are largely
33 unknown. Here, we identify Activin A as an activator of hair cell differentiation and show, using
34 mouse genetic approaches, that a local gradient of Activin A signaling within the auditory
35 sensory epithelium times the longitudinal gradient of hair cell differentiation. Furthermore, we
36 provide evidence that Activin-type signaling regulates a radial gradient of terminal mitosis within
37 the auditory sensory epithelium, which constitutes a novel mechanism for limiting the number of
38 inner hair cells being produced.

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40

41 **INTRODUCTION**

42 Housed in the inner ear cochlea, the auditory sensory organ contains a spiral shaped
43 sensory epithelium specialized to detect and transduce sound. Along its longitudinal axis, two
44 types of mechano-receptor cells, so called inner and outer hair cells, are arranged in distinct
45 rows, with three rows of outer hair cells and a single row of inner hair cells. To ensure the highly
46 stereotyped arrangement of hair cells, cell cycle withdrawal and differentiation within the
47 auditory sensory epithelium occurs in a spatially and temporally highly coordinated manner.
48 Auditory sensory progenitors (pro-sensory cells) exit the cell cycle in an apical-to-basal gradient
49 (Ruben, 1967; Chen and Segil, 1999; Lee et al., 2006), whereas their differentiation into hair
50 cells and supporting cells occurs in an opposing, basal-to-apical gradient (Chen et al., 2002).
51 Previous studies revealed that the morphogen Sonic Hedgehog (SHH) plays a key role in

52 setting up the spatial and temporal pattern of auditory hair cell differentiation. In the
53 undifferentiated cochlea, pro-sensory cells are exposed to high levels of SHH secreted by the
54 adjacent spiral ganglion neurons (SGNs) (Liu et al., 2010; Bok et al., 2013). Ablation of SHH in
55 SGNs or cochlear epithelial-specific ablation of co-receptor Smoothed results in premature
56 hair cell differentiation and in the most extreme case a reversal of the gradient of hair cell
57 differentiation (Bok et al., 2013; Tateya et al., 2013). The differentiation of pro-sensory cells into
58 hair cells is triggered by their upregulation of the basic-helix-loop-helix (bHLH) transcription
59 factor ATOH1, which is both necessary and sufficient for the production of hair cells
60 (Bermingham et al., 1999; Zheng and Gao, 2000; Woods et al., 2004; Cai et al., 2013). SHH
61 signaling opposes hair cell differentiation by maintaining the expression of bHLH transcriptional
62 repressors and ATOH1 antagonists HEY1 and HEY2 within pro-sensory cells (Benito-Gonzalez
63 and Doetzlhofer, 2014). Additional inhibitors of hair cell differentiation belong to the inhibitor of
64 differentiation (ID) family (ID1-4). ID proteins are dominant negative regulators of bHLH
65 transcription factors (Wang and Baker, 2015). Maintained by BMP signaling, ID1, ID2 and ID3
66 are thought to maintain pro-sensory cells in an undifferentiated state by interfering with
67 ATOH1's ability to activate its hair cell-specific target genes (Jones et al., 2006; Kamaid et al.,
68 2010).

69 Much less is known about the signals and factors that promote ATOH1
70 expression/activity within pro-sensory cells and their role in auditory hair cell differentiation.
71 Over-activation of Wnt/ β -catenin signaling has been shown to increase *Atoh1* expression in
72 differentiating cochlear explants and in the absence of Wnt/ β -catenin signaling hair cells fail to
73 form (Jacques et al., 2012; Shi et al., 2014; Munnamalai and Fekete, 2016). However, the
74 pattern of Wnt-reporter activity, which at the onset of hair cell differentiation is high in the
75 cochlear apex but low in the cochlear base, does not parallel the basal-to-apical wave of
76 differentiation (Jacques et al., 2014). Interestingly, the *Inhba* gene, which encodes the Activin A
77 subunit Inhibin β A (Barton et al., 1989), has been shown to be expressed in a basal-to-apical

78 gradient within the differentiating auditory sensory epithelium (Son et al., 2015). Activins, which
79 belong to the transforming growth factor (TGF)- β superfamily of cytokines, control a broad range
80 of biological processes, including reproduction, embryonic axial specification, organogenesis
81 and adult tissue homeostasis (reviewed in (Namwanje and Brown, 2016)). In the developing
82 spinal cord, Activins and other TGF- β -related ligands are required in most dorsally located
83 neuronal progenitors for *Atoh1* induction and their subsequent differentiation as D1A/B
84 commissural neurons (Lee et al., 1998; Wine-Lee et al., 2004). The role of Activins in *Atoh1*
85 regulation and hair cell differentiation are currently unknown.

86 Here, we identify Activin A signaling as a positive regulator of *Atoh1* gene expression
87 and hair cell differentiation. We find that its graded pattern of activity within the auditory sensory
88 epithelium times the basal-to-apical wave of hair cell differentiation. Furthermore, we provide
89 evidence that Activin signaling informs a previously unidentified medial-to-lateral gradient of
90 terminal mitosis that forces inner hair cell progenitors located at the medial edge of the sensory
91 epithelium to withdraw from the cell cycle prior to outer hair cell progenitors.

92 93 **RESULTS**

94 95 **The graded pattern of Activin A expression parallels auditory hair cell differentiation.**

96 The biological activity of Activin A and other Activins is limited by the secreted protein
97 Follistatin (FST). Two FST molecules encircle the Inhibin β dimer, blocking both type I and type
98 II receptor binding sites, thus preventing receptor binding and activation of its downstream
99 signaling cascade (Thompson et al., 2005). Within the differentiating auditory sensory
100 epithelium *Fst* and the Inhibin β A encoding gene *Inhba* are expressed in opposing gradients,
101 with *Inhba* being highest expressed within the basal sensory epithelium and *Fst* being highest
102 expressed apically (Son et al., 2015). To further characterize the pattern of *Inhba* and *Fst*
103 expression and explore a potential correlation with hair cell differentiation, we carried out RNA in
104 situ hybridization experiments using cochlear tissue stages E13.5-E15.5. *Sox2* expression was

105 used to identify the pro-sensory/sensory domain, containing hair cell and supporting cell
106 progenitors/precursors (Fig. 1J-L). Nascent hair cells were identified by their expression of
107 *Atoh1* (Fig.1 A-C). In mice, differentiation of basally located pro-sensory cells into hair cells
108 starts at around embryonic stage E13.5-E14.0 (Fig.1 A). Paralleling *Atoh1* expression, *Inhba*
109 expression was limited to the basal pro-sensory domain (Fig.1 D). In contrast, *Fst* was highly
110 expressed within the lateral part of the pro-sensory domain throughout the cochlear apex and
111 mid turn, but was only weakly expressed in the cochlear base (Fig. 1 G). At stages E14.5 and
112 E15.5, as hair cell differentiation progressed towards the cochlear apex (Fig. 1 B, C), *Inhba*
113 expression within the pro-sensory/sensory domain extended to the cochlear mid-turn (Fig.1 E,
114 F). At the same time, *Fst* expression further regressed in the cochlear base and weakened in
115 the cochlear mid-turn, but continued to be highly expressed in the undifferentiated cochlear
116 apex (Fig. 1 H, I). As summarized in Fig.1 M, our analysis of *Inhba* and *Fst* expression indicates
117 the existence of a basal-to-apical and medial-to-lateral gradient of Activin A signaling within the
118 differentiating auditory sensory epithelium, which closely resembles the spatial and temporal
119 pattern of hair cell differentiation, suggesting a link between Activin A signaling and hair cell
120 differentiation.

121

122 **Activin signaling promotes auditory hair cell differentiation.**

123 Activins play a central role in coordinating growth and differentiation in sensory and
124 neuronal tissues, including the spinal cord, retina and olfactory epithelium (Liem et al., 1997;
125 Davis et al., 2000; Gokoffski et al., 2011). To determine the role of Activin A in cochlear hair cell
126 differentiation, we exposed undifferentiated cochlear tissue (stage E13.5) to recombinant Activin
127 A (500 ng/ml) for 24 hours (Fig. 2 A). To monitor the dynamics of hair cell differentiation, we
128 made use of transgenic mice that carried the enhanced green fluorescent protein (EGFP)
129 transgene under the control of the *Atoh1* enhancer (Lumpkin et al., 2003). Hair cell
130 differentiation follows a steep basal-to-apical gradient in which hair cells located within the

131 cochlear mid-basal segment differentiate prior to more apical located hair cells. In addition, a
132 shallower, medial-to-lateral gradient exists, with inner hair cells differentiating prior to their
133 neighboring more laterally located outer hair cells (Chen et al., 2002). Whereas control cultures
134 showed no or only weak *Atoh1*-reporter activity (EGFP) within the developing cochlear duct
135 after 12 hours of culture (Fig. 2 B), Activin A-treated cochlear cultures already contained a
136 narrow strip of EGFP positive inner hair cells (Fig. 2 C). 6 hours later, a broad strip of EGFP
137 positive cells representing inner and outer hair cells was evident in Activin A-treated cochlear
138 explants (Fig. 2 E), whereas control cochlear explants contained only few scattered EGFP
139 positive inner hair cells (Fig. 2 D). Furthermore, at all stages examined, the band of EGFP
140 positive cells extended further apically in Activin A treated cochlear explants compared to
141 control (Fig. 2 B-G, H). Taken together our findings indicate that Activin A acts a differentiation
142 signaling for auditory hair cells.

143 To identify target genes of Activin signaling in the developing cochlea, we cultured E13.5
144 cochlear explants with or without Activin A for 24 hours, after which we enzymatically purified
145 the corresponding cochlear epithelia and performed RT-qPCR. Our analysis focused on key
146 regulators of hair cell differentiation, which included the pro-hair cell factor *ATOH1*, the sensory
147 specification factor *SOX2* (Neves et al., 2012; Kempfle et al., 2016), the hair cell fate repressors
148 *HEY1*, *HEY2* (Benito-Gonzalez and Doetzlhofer, 2014), as well as members of the inhibitor of
149 differentiation (ID) family (*ID1-4*). We found that Activin A treatment did not significantly alter
150 mRNA abundance of *Hey1*, *Hey2*, *Sox2*, *Id1* and *Id2* expression. However, we found that in
151 response to Activin treatment *Atoh1* transcript was significantly increased by more than 2-fold
152 and that *Id3* and *Id4* mRNA abundance was significantly reduced (Fig. 2 I).

153 To untangle direct and indirect effects of Activin signaling on *Id3*, *Id4* and *Atoh1* gene
154 expression we shortened the culture period to 3.5 hours and eliminated non-epithelial tissue
155 from our cultures. In addition, we included a brief BMP4 treatment as positive control, allowing
156 us to compare the effects of Activin A and BMP4 on pro-sensory/sensory gene expression.

157 BMP signaling is known to positively regulate *Id1-3* expression in inner ear pro-sensory cells
158 (Kamaid et al., 2010; Ohyama et al., 2010). Consistent with these previous reports, 3.5 hour
159 BMP4 treatment of E13.5 cochlear epithelia led to an increase in *Id1-3* expression, which was
160 significant for *Id1* and *Id3*, but showed no effect on *Id4* expression compared to control (Fig. 2
161 K). In contrast, 3.5 hour Activin A treatment of E13.5 cochlear epithelia had no effect on *Id1-3*
162 expression but resulted in a modest, but significant decrease in *Id4* expression compared to
163 control (Fig. 2 K). The divergent transcriptional responses to Activin A and BMP4 are likely the
164 consequence of differences in type I receptor and R-SMAD utilization. Activins commonly utilize
165 the type I receptor ALK4 (ACVR1B), which signals through SMAD2 and SMAD3, whereas
166 BMPs activate the type I receptors ALK3 (BMPR1A) and ALK6 (BMPR1B), which signal through
167 SMAD1, SMAD5 and SMAD9 activation (reviewed in (Miyazawa et al., 2002)). Indeed, we found
168 that a 3.5-hour exposure of E13.5 cochlear epithelia to Activin A selectively stimulated the
169 phosphorylation of SMAD2/3, whereas a 3.5-hour treatment with BMP4 resulted in the
170 phosphorylation of SMAD1/5/9 (Fig. 2 J, K). In contrast to the 24-hour Activin A treatment, the
171 3.5-hour Activin A treatment failed to significantly increase *Atoh1* expression, suggesting that
172 Activin A promotes the expression of *Atoh1* through indirect mechanisms.

173

174 **FST inhibits auditory hair cell differentiation**

175 To characterize the function of FST in cochlear development we made use of a recently
176 developed doxycycline (dox) inducible transgenic mouse line, in which a cassette encoding the
177 human FST-288 isoform is under the control of a tetracycline-responsive promoter element
178 (tetO) (Lee and McPherron, 2001; Roby et al., 2012) (Fig. 3 A). In the presence of a ubiquitously
179 expressed reverse tetracycline-controlled trans-activator (rtTA), dox administration allows for
180 robust induction of the human (h) FST transgene (Fig. 3 A, B, G). To assess the role of FST in
181 cochlear hair cell differentiation, timed pregnant females received dox beginning at E11.5 and
182 double transgenic FST over-expressing embryos (R26-FST) and single transgenic littermates

183 lacking the R26-M2rtTA transgene (control), were harvested three days later (E14.5). Inclusion
184 of the *Atoh1*-reporter transgene (*Atoh1/nEGFP*) allowed for the ready analysis of hair cell
185 differentiation at the time of isolation. While *Atoh1*-reporter positive inner hair cells were
186 detectable in the cochlear base of stage E14.5 control cochlear tissue (Fig. 3 C, E), stage E14.5
187 FST overexpressing cochlear tissue lacked cochlear hair cells (Fig. 3 D, F), indicating an
188 inhibitory function for FST in auditory hair cell differentiation.

189 To determine how FST overexpression interferes with hair cell differentiation we again
190 administered dox at E11.5, isolated FST transgenic and cochlear epithelia from stage E14.5
191 FST overexpressing and control embryos and analyze their pro-sensory/ sensory gene
192 expression using RT-qPCR. We found that FST overexpression significantly reduced *Atoh1*
193 expression and significantly increased *Hes1*, *Id3* and *Id4* expression, but did not significantly
194 alter *p27/Kip1*, *Id2* or *Hey1* expression (Fig. 3 H). Activins as well as the Activin-type ligands
195 Gdf11 and myostatin are high affinity binding partners for FST (Harrington et al., 2006). In
196 addition, FST has been shown to bind at low affinity to BMP4 and BMP7 and modulate their
197 activity (Iemura et al., 1998; Amthor et al., 2002). These two related BMP ligands are
198 abundantly expressed in the developing cochlea and are thought to function in the specification
199 and patterning of the pro-sensory domain (Ohyama et al., 2010). To determine whether FST
200 overexpression inhibits BMP signaling in the developing cochlea, we administered dox at E12.5,
201 and two days analyzed P-SMAD2/3 and P-SMAD1/5/9 proteins in acutely isolated control and
202 FST overexpressing cochlear epithelia. Our western blot analysis revealed that P-SMAD2/3
203 protein levels were reduced in FST overexpressing cochlear epithelia compared to control
204 cochlear epithelia. However, FST overexpression had little to no effect on P-SMAD1/5/9 protein
205 levels, indicating that FST overexpression in the developing cochlea selectively disrupts Activin-
206 type signaling (Fig. 3 E).

207 To determine whether exogenous Activin A can rescue the FST mediated delay in hair
208 cell differentiation, we monitored *Atoh1*-reporter expression in cultures of E13.5 FST

209 overexpressing (R26-FST) and single transgenic wild type cochlear tissue treated with or
210 without Activin A (Fig. 4 A). Consistent with our earlier findings, we observed that hair cell
211 differentiation occurred significantly earlier in Activin A-treated wild type cochlear explants
212 compared to untreated wild type cochlear explants (Fig. 4 C, B, N). Conversely, the onset of hair
213 cell differentiation was significantly delayed in untreated FST overexpressing cochlear explants
214 compared to untreated wild type cochlear explants (Fig. 4 F, H, N). However, these defects
215 were almost completely abolished when Activin A treatment and FST overexpression were
216 combined and no significant differences in the onset (Fig. 4 F, I, N) or in the progression of hair
217 cell differentiation was observed between FST overexpressing cochlear explants treated with
218 Activin A and wild type untreated cochlear explants (Fig. 4 J, M, N). Taken together, our data
219 suggests that FST antagonizes hair cell differentiation in an Activin A-dependent manner.

220

221 **FST promotes pro-sensory cell proliferation.**

222 In the mammalian cochlea, cell cycle exit of pro-sensory cells occurs in a highly
223 synchronized manner. Pro-sensory cells located in the cochlear apex withdraw from the cell
224 cycle first, followed by more basally located pro-sensory cells (Ruben, 1967; Lee et al., 2006).
225 To determine whether FST antagonizes pro-sensory cell cycle withdrawal, we injected timed
226 pregnant dams with the thymidine analog EdU at stage E13.5, which in mice corresponds to the
227 peak of pro-sensory cell cycle withdrawal, and analyzed hair cell and supporting cell-specific
228 EdU incorporation in FST overexpressing embryos and control littermates five days later (stage
229 E18.5). Hair cells were identified by their native EGFP (Atoh1/nEGFP) expression as well as by
230 immuno-staining for the hair cell-specific protein myosin VIIa (Myo7a); supporting cells were
231 identified by SOX2 immuno-staining. Classification of cell subtypes was based on their location
232 within the sensory epithelium. As expected we found that basally located hair cells and their
233 surrounding supporting cells incorporated EdU at high frequency in both control (Fig. 5 A) and
234 FST overexpressing cochlear tissue (Fig. 5 D). However, whereas in control cochlear tissue

235 supporting cells and hair cells located further apically (mid and apex) showed little to no EdU
236 incorporation (Fig. 5 B, C), their counterparts in FST overexpressing cochleae exhibited robust
237 EdU incorporation (Fig. 5 E, F), indicating that FST overexpression in the developing cochlea
238 delays pro-sensory cell cycle withdrawal. The number and percentage of EdU labeled inner and
239 outer hair cells was significantly lower in the apex than in the mid-turn of FST overexpressing
240 cochleae, indicating that FST overexpression prolongs pro-sensory cell proliferation without
241 disrupting the apical-to-basal gradient of pro-sensory cell cycle withdrawal (Fig. 5 G, H). Our
242 analysis did however find that FST overexpression alters a previously unrecognized medial-to-
243 lateral gradient of terminal mitosis within the auditory sensory epithelium. In the basal segment
244 of the control and FST overexpressing auditory sensory epithelia, in which, at the time of EdU
245 administration pro-sensory cells had been actively dividing, the percentage of EdU positive hair
246 cells that were inner hair cells correlated with their relative abundance (Fig. 5 I, base). However,
247 in the mid-turn of control cochleae, where pro-sensory cells had been in the process of
248 withdrawing from the cell cycle, no EdU positive inner hair cells were detected and 100% of the
249 EdU labeled hair cells were outer hair cells, suggesting that inner hair cell progenitors had
250 exited the cell cycle prior to the more laterally located outer hair cell progenitors. However, in
251 FST overexpressing cochleae this radial gradient of terminal mitosis appeared to be lost (Fig. 5
252 I, mid).

253 To further characterize the effects of FST on inner hair cell progenitor proliferation, we
254 administered EdU from E14.5 to E17.5 and analyzed EdU incorporation in hair cells and
255 supporting cells at E18.5. In control cochlear tissue, only few, most basally located hair cells
256 and supporting cells were labeled with EdU (Fig. 6 A-C, G, H), indicating that the majority of pro-
257 sensory cells had withdrawn from the cell cycle prior to stage E14.5. In contrast, robust EdU
258 labeling of supporting cells and hair cells was observed throughout the base and mid-turn of
259 FST overexpressing cochleae (Fig. 6 D-F, G, H). Again, in control tissue inner hair cells that
260 were inner hair cells was increased in FST overexpressing cochlear tissue compared to control

261 Consistent with the existence of a medial to lateral gradient of cell cycle withdrawal, in the base
262 of control cochlear tissue, 94 % of all EdU labeled hair cells were outer hair cells and only 6% of
263 all EdU labeled hair cells were inner hair cells. In contrast, in the base of FST overexpressing
264 cochlear tissue 51% of all EdU labeled hair cells were inner hair cells (Fig. 6 I). In the apex of
265 FST overexpressing cochleae, inner hair cells and inner phalangeal cells were the only cell
266 types that were labeled with EdU (Fig. 6 F, G, H). In summary, our findings indicate that FST
267 acts as a mitogen for pro-sensory cells, in particular inner hair cell progenitors and identify FST
268 as a key regulator of a newly uncovered radial gradient of pro-sensory cell cycle withdrawal.

269

270 **Activin signaling controls the differentiation and cellular patterning of hair cells.**

271 As typical for wild type cochlear tissue, cochlear tissue from stage E18.5 control
272 embryos contained a single row of inner hair cells and 3 rows of outer hair cells (Fig. 7 A, C, E).
273 However, likely a consequence of continued proliferation of inner hair cell progenitors, cochlear
274 tissue from FST overexpressing embryos contained 2-3 rows of inner hair cells. The ectopic
275 inner hair cell phenotype was most severe in the base of the cochlea; inner hair cell density was
276 increased nearly 2.5-fold compared to controls and was accompanied by an increase in
277 surrounding inner phalangeal cells (Fig. 7 B, D, G, H). The density and cellular patterning of
278 outer hair cells was largely unchanged in FST overexpressing cochleae except for in the
279 cochlear base. The base of FST overexpressing cochleae contained stretches of sensory
280 epithelium in which the 3rd row of outer hair cells and the 3rd row of accompanying Deiters' cells
281 were missing, resulting in reduced outer hair cell and Deiters' cell densities (Fig. 7 D, G, H).
282 Furthermore, differentiation/maturation of the auditory sensory epithelium was severely delayed
283 in response to FST overexpression. At stage E18.5, the basal-to-apical wave of differentiation
284 has reached the cochlear apex. The initially multilayered auditory sensory epithelium is largely
285 thinned to a two-layered sensory epithelium and is near its final length. Moreover, hair cells,
286 including hair cells located in the cochlear apex, have formed actin-rich apical protrusions, so

287 called stereocilia (see Fig. 7 C, E). Consistent with a severe delay in pro-sensory cell
288 differentiation, hair cells in FST overexpressing cochlear tissue had less developed stereocilia
289 than their wild type counterparts, and apical hair cells lacked stereocilia completely (Fig. 7 C-F).
290 Moreover, our analysis revealed that the auditory sensory epithelia of FST overexpressing
291 cochleae consisted of more than two epithelial layers (see Fig. 5 A-F) and were 30% shorter
292 compared to control sensory epithelia obtained from littermates (dox E11.5, harvest E18.5:
293 control = 5.14 ± 0.04 mm, R26-FST = 3.65 ± 0.13 mm, $n=5$, $p=0.0002$).

294 FST induction one day later at ~ E13.0 (dox E12.5), had no effect on the length of the
295 auditory sensory epithelium (dox E12.5, harvest E17.5: control = 4.13 ± 0.17 mm, R36-FST
296 = 3.99 ± 0.12 mm, $n=3$, $p=0.5425$), but we continued to observe ectopic inner hair cells in FST
297 overexpressing cochleae, with the highest number of ectopic hair cells found in the basal portion
298 of the auditory sensory epithelium, as well as short stretches of sensory epithelium with only 2
299 rows of outer hair cells (Fig. 7 J, M). Furthermore, we found that hair cell stereocilia in FST
300 overexpressing cochlea had a less mature phenotype compared to their counterparts in control
301 cochlear tissue, indicating that FST induction as late as ~E13.0 causes a delay in hair cells
302 differentiation/maturation (Fig. 7 I-L).

303 The formation of inner hair cells is regulated by a complex interplay of various signaling
304 pathways including Notch, Wnt and BMP signaling (reviewed in (Groves and Fekete, 2012)). In
305 particular, a recent study revealed that inhibition of BMP signaling using dorsomorphin results in
306 the overproduction of inner hair cells in cochlear explants (Munnamalai and Fekete, 2016).

307 To determine whether FST influences inner hair cell formation in an Activin A-dependent
308 manner, we analyzed hair cell patterning in Activin A (*Inhba*) mutant mice. To selectively ablate
309 *Inhba* gene function in the developing inner ear, we intercrossed *Inhba* floxed mice (*Inhba* fl/fl),
310 in which exon 2 of the *Inhba* gene is flanked by LoxP sites (Pangas et al., 2007), with inner ear-
311 specific *Pax2-Cre* transgenic mice (Ohyama and Groves, 2004). Conditional knockout (cKO) of
312 *Inhba* gene function did not alter overall cochlear morphology, nor did it alter the length of the

313 sensory epithelium compared to control (*Inhba* fl/fl) (P0: control = 4.99 ± 0.12 mm; *Inhba* cKO =
314 4.82 ± 0.01 mm; n=2, p-value=0.4043). However, our analysis revealed defects in inner hair
315 cell patterning in the absence of *Inhba* that were qualitatively similar to inner hair cell patterning
316 defects observed in response to FST overexpression (dox E12.5). Cochlear tissue from control
317 littermates (*Inhba* fl/fl) contained the normal compliment of one row of inner hair cells and three
318 rows of outer hair cells (Fig. 7 N-P). In contrast, *Inhba* cKO (*Pax2-Cre*; *Inhba* fl/fl) cochlear
319 tissue contained ectopic inner hair cells, with much of the ectopic inner hair cells residing within
320 the most basal segment (Fig. 7 Q-S, T). Furthermore, consistent with a delayed onset of hair
321 cell differentiation, hair cell stereocilia in the base, mid and apex of *Inhba* mutant cochlear tissue
322 had a less mature phenotype compared to control cochlear tissue (Fig. 5 N-S). In summary,
323 these findings indicate that Activin A-mediated signaling is critical for proper inner hair cell
324 formation as well as hair cell differentiation/maturation *in vivo*.

325

326 **DISCUSSION**

327 Signaling gradients play a fundamental role in controlling growth and differentiation
328 during embryonic development. Here, we show that Activin A acts as differentiation signal for
329 auditory hair cells and provide evidence that its graded activity times the longitudinal gradient of
330 differentiation within the auditory sensory epithelium. Furthermore, we identify FST as an
331 antagonist of Activin signaling and show that FST overexpression delays pro-sensory cell cycle
332 exit and differentiation. Finally, we provide evidence that Activin signaling regulates a previously
333 unrecognized radial gradient of pro-sensory cell cycle withdrawal, limiting the number of inner
334 hair cells being produced.

335

336 **Activin A functions as a pro-differentiation signal in the mammalian cochlea.**

337 How does Activin signaling promote auditory hair cell differentiation? Our findings
338 suggest that Activin A facilitates hair cell differentiation through boosting the expression of the

339 pro-hair cell transcription factor ATOH1 within pro-sensory cells. The time required to observe
340 robust increase in *Atoh1* expression in response to exogenous Activin A suggests that Activin A
341 promotes *Atoh1* expression through indirect mechanisms. In particular, we found that Activin
342 signaling reduced the expression of bHLH antagonist ID3 and ID4 within pro-sensory cells. ID
343 proteins function as dominant-negative regulators of bHLH transcription factors by binding to
344 bHLH E-proteins, which are required for bHLH transcription factors such as ATOH1 to form
345 functional hetero-dimers capable of binding DNA (Benezra et al., 1990; Wang and Baker, 2015).
346 Consistent with acting as an ATOH1 antagonist, ID3 overexpression in the developing inner ear
347 inhibits hair cell formation in both auditory and vestibular sensory organs (Jones et al., 2006;
348 Kamaid et al., 2010). The role of ID4 in inner ear development has yet to be established.

349 It should be noted that *Atoh1* expression and subsequent hair cell differentiation is not
350 disrupted, but rather delayed in response to FST overexpression or *Inhba* ablation, suggesting
351 the existence of Activin-independent mechanisms capable of activating *Atoh1* expression in pro-
352 sensory cells. A likely candidate is the Wnt/ β -catenin signaling pathway. The Wnt effector β -
353 catenin is required for hair cell specification and has been shown to directly activate *Atoh1*
354 transcription in neuronal progenitors (Shi et al., 2010; Shi et al., 2014). Our here presented data
355 is most consistent with a model in which Wnt/ β -catenin signaling is required to initiate *Atoh1*
356 expression, whereas Activin signaling functions to enhance *Atoh1* expression/activity, providing
357 spatial and temporal control over hair cell differentiation. Wnt/ β -catenin signaling and TGF- β -
358 related pathways are known to cooperate with the transcriptional regulation of their target genes
359 (Labbe et al., 2000) (Luo, 2017) and it will be of interest to resolve whether and how Activin and
360 Wnt signaling pathways collaborate in *Atoh1* gene regulation. Furthermore, it will be of interest
361 to identify the upstream signals/factors that induce *Inhba* expression at the onset of hair cell
362 differentiation. A potential candidate is the Notch signaling pathway. Notch signaling is active in
363 pro-sensory cells and has been recently shown to positively regulate *Inhba* expression in

364 differentiating and terminal differentiated supporting cells (Campbell et al., 2016; Maass et al.,
365 2016).

366 **FST maintains pro-sensory cells in an undifferentiated and proliferative state.** We

367 show that FST overexpression severely delays the onset of pro-sensory cell cycle withdrawal

368 and differentiation. A similar function in timing pro-sensory cell withdrawal and differentiation

369 has been recently reported for the RNA binding protein LIN28B (Golden et al., 2015).

370 Interestingly, the expression of *Fst* in the developing cochlea largely mimics that of *Lin28b*; each

371 are initially highly expressed in pro-sensory cells and are downregulated upon differentiation

372 following a basal-to-apical gradient. Regulatory and functional connections between Activin

373 signaling and LIN28B have not yet been established and it will be of interest to determine

374 whether a link between LIN28B and FST exists. TGF β -related signaling pathways are known to

375 inhibit proliferation by increasing the expression and/or activity of cyclin dependent kinase

376 (CDK) inhibitors (Massague and Gomis, 2006). In the developing cochlea, the CDK inhibitor

377 p27/Kip1 (CDKN1B) is the main regulator of pro-sensory cell cycle exit and its transcriptional

378 upregulation directs the apical-to-basal wave of pro-sensory cell cycle withdrawal (Chen and

379 Segil, 1999; Lee et al., 2006). However, we found no evidence that FST overexpression

380 interfered with the transcriptional regulation of *p27/Kip1*, suggesting that the observed delay in

381 pro-sensory cell cycle exit was likely caused by a reduction in P27/Kip1 protein stability and/or

382 activity. In addition, it is likely that the increase in *Id3* and *Id4* expression in response to FST

383 overexpression contributed to the observed delay in pro-sensory cell cycle exit. High ID protein

384 expression is associated with a proliferative, undifferentiated cell state and ID4 expression in

385 spermatogonia and mammary glands is a predictor for stemness (Wang and Baker, 2015;

386 Hesel et al., 2017).

387

388 **A radial Activin-FST counter gradient controls the production of inner hair cells.**

389 Interestingly, we find that FST overexpression and to a lesser extent ablation of Activin
390 A (*Inhba*) results in an overproduction of inner hair cells. Based on our findings we propose that
391 FST overexpression disrupts a previously unrecognized radial gradient of pro-sensory cell cycle
392 withdrawal, leading to prolonged inner hair cell progenitor proliferation. We provide evidence for
393 the existence of a radial (medial-to-lateral) gradient of Activin A activity. We show that at the
394 peak of terminal mitosis (E13.5), as *Inhba* expression is induced within the basal pro-sensory
395 domain, *Fst* expression is maintained at the lateral edge of the pro-sensory domain, creating a
396 medial-to-lateral gradient of Activin A signaling. Furthermore, we show that in wild type tissue
397 inner hair cell progenitors withdraw from the cell cycle prior to outer hair cell progenitor cells and
398 demonstrate that this distinct medial-to-lateral gradient of pro-sensory cell cycle withdrawal is
399 disrupted in response to FST overexpression. This newly identified radial gradient of terminal
400 mitosis constitutes a novel mechanism for limiting the number of inner hair cells being produced
401 in the mammalian cochlea.

402

403 **METHODS**

404 **Experimental animals.** *Atoh1/nEGFP* transgenic mice (Lumpkin et al., 2003) were obtained
405 from Jane Johnson, University of Texas, Southwestern Medical Center. *Pax2-Cre* BAC
406 transgenic mice (Ohyama and Groves, 2004) were obtained from Andrew Groves, Baylor
407 College. *Inhba* floxed mice (Pangas et al., 2007) were obtained from Martin Matzuk, Baylor
408 College. The R26-M2rtTA (Hochedlinger et al., 2005)(stock no. 006965) was purchased from
409 Jackson Laboratories (Bar Harbor, ME). FST transgenic mice were obtained from Se-Jin Lee,
410 Johns Hopkins University, School of Medicine. In this line a cassette encoding the human FST-
411 288 isoform is under the control of a tetracycline-responsive promoter element (tetO) (Lee,
412 2007; Roby et al., 2012). Mice were genotyped by PCR. *Pax2-Cre*: Cre1F
413 (GCCTGCATTACCGGTCGATGCAACGA), *Cre1R* (GTGGCAGATGGCGCGGCAACACCATT)
414 yields a 700bp band. *Inhba* floxed: *Inhba* fx1 (AAG AGA GAA TGG TGT ACC TTC ATT), *Inhba*

415 fx2 (TAT AAC CTG GGT AAG TGG GT), Inhba fx3 (AGA CGT GCT ACT TCC ATT TG) yield a
416 400bp band for the floxed allele and a 280bp for the wild type allele. R26-M2rtTA: MTR (GCG
417 AAG AGT TTG TCC TCA ACC), F (AAA GTC GCT CTG AGT TGT TAT), WTR (GGA GCG
418 GGA GAA ATG GAT ATG) yield a 340bp band for the mutant allele and a 650bp for the wild
419 type allele. FST: YA88 (TTGCCTCCTGCTGCTGCTGC), YA123
420 (TTTTTCCCAGGTCCACAGTCCACG) yields a 247bp band for the FST transgene.
421 Atoh1/nGFP: EGFP1 (CGA AGG CTA CGT CCA GGA GCG CAC), EGFP2 (GCA CGG GGC
422 CGT CGC CGA TGG GGG TGT) yields a 300bp band for EGFP. Mice were maintained on a
423 C57BL/6; CD-1 mixed background. Mice of both sexes were used in this study. Embryonic
424 development was considered as E0.5 on the day a mating plug was observed. To induce *FST*
425 transgene expression doxycycline (dox) was delivered to time-mated females via ad libitum
426 access to feed containing 2 grams of dox per kilogram feed (Bioserv no. F2893). All
427 experiments and procedures were approved by the Johns Hopkins University Institutional
428 Animal Care and Use Committee (protocol #MO17M318), and all experiments and procedures
429 adhered to the National Institutes of Health-approved standards.

430

431 **Tissue harvest and processing.** Embryos and early postnatal pups were staged using the
432 EMAP eMouse Atlas Project (<http://www.emouseatlas.org>) Theiler staging criteria. Inner ear
433 cochleae were collected in Hanks buffer (Corning Cellgro). To free the cochlear epithelial duct
434 from surrounding tissue, dispase (1mg/ml; Invitrogen) and collagenase (1mg/ml; Worthington)
435 mediated digest was used as previously described (Golden et al., 2015). To obtain cochlear
436 whole mount preparations (also referred to as surface preparations) containing the auditory
437 sensory epithelium (E18.5-P0), the cochlear capsule, spiral ganglion, and Reissner's membrane
438 were removed, and the remaining tissue was briefly fixed in 4% (vol/vol) paraformaldehyde
439 (PFA) (Electron Microscopy Sciences) in PBS. To obtain cochlear sections, heads were fixed in
440 4% PFA in PBS, cryoprotected using 30% sucrose in PBS, and embedded in OCT (Sakura

441 Finetek). Tissue was sectioned at a thickness of 14 μ m and collected on SuperFrost Plus slides
442 (Thermo Scientific) and stored at -80°C.

443

444 **Histochemistry and in situ hybridization.** Immunostaining was performed according to the
445 manufacturer's specifications. Primary antibodies: rabbit anti-myosinVIIa (1:500, Proteus no.
446 25–6790), goat anti-SOX2 (1:500, Santa Cruz no. sc-17320). Cell nuclei were fluorescently
447 labeled with Hoechst-33258 dye (Sigma). Actin filaments were labeled with Alexa Fluor (488 or
448 546) conjugated phalloidin (1:1000, Invitrogen). Alexa Fluor (488 or 546) labeled secondary
449 antibodies (1:1000, Invitrogen) were used. For insitu hybridization digoxigenin (DIG)-labeled
450 antisense RNA probes were prepared according to the manufacturer's specifications (Roche).
451 PCR amplified fragments of *Inhba* (NM_008380, 47-472) and *Fst* (NM_008046, 180-492) were
452 used as template and gene-specific T7 RNA polymerase promoter hybrid primers were used for
453 in vitro transcription. *Atoh1* (NM_007500) and *Sox2* (NM_011443) probes were prepared as
454 previously described (Golden et al., 2015). Probes were detected with the anti-DIG-AP (alkaline
455 phosphatase) conjugated antibody (Roche), and the color reactions were developed by using
456 BM Purple AP Substrate (Roche).

457

458 **Cochlear explant culture and hair cell differentiation assay.** Wild type or FST
459 overexpressing embryos and their control littermates were screened for native EGFP
460 (*Atoh1*/nEGFP) expression and staged (see tissue harvest and processing). Embryos of
461 inappropriate stage and *Atoh1*/nEGFP negative embryos were discarded. Cochleae from
462 individual embryos were harvested in Hanks media (Life Technologies), and treated with
463 dispase and collagenase to remove cochlear capsule. The remaining tissue, including the
464 cochlear epithelial duct, the vestibular sacculus, and the innervating spiral ganglion, was placed
465 onto filter membranes (SPI Supplies, Structure Probe) and cultured in DMEM-F12 (Life
466 Technologies), 1% FBS (Atlanta Biologicals), 5 ng/ml EGF (Sigma), 100 U/ml penicillin-

467 streptomycin (Sigma), and 1xB27 supplement (Life Technologies). Activin A (final conc.
468 500ng/ml) was added at plating and was replenished daily. All cultures were maintained in a 5%
469 CO₂ / 20% O₂ humidified incubator. To monitor hair cell differentiation, green fluorescent images
470 of native EGFP expression were captured using fluorescent stereo-microscopy (Leica).
471 Fluorescent images were analyzed in Photoshop CS6 (Adobe), and lengths of Atoh1/nEGFP-
472 positive domains were measured using ImageJ software (National Institutes of Health).

473

474 **Recombinant protein:** Recombinant human/mouse/rat Activin A (R&D Systems no. 338-AC)
475 was reconstituted in sterile PBS containing 0.1% BSA at a concentration of 50 µg/ml and used
476 at final concentration of 200-500 ng/ml. Recombinant human BMP4 (R&D Systems, no. 314-BP-
477 010) was reconstituted in sterile 4 mM HCl 0.1% BSA at a concentration of 100 µg/ml and used
478 at 100 ng/ml final concentration. Stock solutions were stored at -80°C.

479

480 **RNA extraction and q-PCR.** Cochlear epithelia were isolated from cultured cochlear explants
481 or freshly harvested inner ear tissue using dispase/collagenase treatment. RNeasy Micro kit
482 (Qiagen) was used to isolate total RNA, and mRNA was transcribed into cDNA using iScript kit
483 (Bio-Rad). Q-PCR was performed with Fast SYBR Green Master Mix reagent (Applied
484 Biosystems) and gene-specific primer sets on a CFX-Connect Real Time PCR Detection
485 System (Bio-Rad). Each PCR was performed in triplicate. Relative gene expression was
486 analyzed by using the $\Delta\Delta CT$ method (Schmittgen and Livak, 2008). The ribosomal gene Rpl19
487 was used as endogenous reference gene. The following q-PCR primers were used:

488	Gene	Forward Primer	Reverse Primer
489	Atoh1	ATG CAC GGG CTG AAC CA	TCG TTG TTG AAG GAC GGG ATA
490	Hey1	CAC TGC AGG AGG GAA AGG TTA T	CCC CAA ACT CCG ATA GTC CAT
491	Hey2	AAG CGC CCT TGT GAG GAA A	TCG CTC CCC ACG TCG AT
492	Hes1	GCT TCA GCG AGT GCA TGA AC	CGG TGT TAA CGC CCT CAC A

493 Hes6 CCA TCG ATG CCA CTG TCT CA GCA GCG GCA TGG ATT CTA G
494 Id1 GAA CGT CCT GCT CTA CGA CAT G TGG GCA CCA GCT CCT TGA
495 Id2 AAG GTG ACC AAG ATG GAA ATC CT CGA TCT GCA GGT CCA AGA TGT
496 Id3 GAG CTC ACT CCG GAA CTT GTG CGG GTC AGT GGC AAA AGC
497 Id4 TGC GAT ATG AAC GAC TGC TAC A TTG TTG GGC GGG ATG GTA
498 P27 GCA GGA GAG CCA GGA TGT CA CCT GGA CAC TGC TCC GCT AA
499 Ptch1 CTG GCT CTG ATG ACC GTT GA GCA CTC AGC TTG ATC CCA ATG
500 Rpl19 GGT CTG GTT GGA TCC CAA TG CCC GGG AAT GGA CAG TCA
501 Sox2 CTG TTT TTT CAT CCC AAT TGC A CGG AGA TCT GGC GGA GAA TA

502

503 **Western blot.** Individual cochlear epithelia were lysed in RIPA lysis buffer (Sigma)
504 supplemented with Roche Protease Inhibitor (Sigma) and Phosphatase Inhibitor Cocktail no.2
505 and no.3 (Sigma). Following manufactures recommendations, equal amounts of cochlear
506 protein extract were resolved on NuPAGE 4-12% Bis-Tris Gels (Invitrogen) and transferred to
507 Immun-Blot PVDF membrane (Bio-rad) by electrophoresis. Membranes were blocked in 5% no-
508 fat dry milk in TBST and immunoblotted with rabbit anti-P-Smad2/3 (1:1,000 Cell Signaling,
509 no.8828), P-Smad1/5/9 1:1,000 (Cell Signaling, no.13820) and mouse anti- β -actin 1:1,000
510 (Santa Cruz, no.SC-47778). HRP-conjugated secondary antibodies from Jackson Immuno
511 Research were used at a concentration of 1:10,000 (goat anti-rabbit IgG, no.111-035-003;
512 sheep anti-mouse IgG no.515-035-003). Signal was revealed using a Western Lightening-ECL
513 kit (Perkin Elmer) or SuperSignal West Femto Maximum Sensitivity Substrate (Thermo
514 Scientific) according to manufacturer's instructions.

515

516 **Quantification of hair cells and supporting cells.** Cell counts were performed in cochlear
517 whole mounts. Hair cells were identified by their native EGFP (Atoh1/nEGFP) expression, as

518 well as by immuno-staining for the hair cell-specific protein myosinVIIa (Myo7a); supporting cells
519 were identified by SOX2 immuno-staining. Classification of cell subtypes was based on their
520 location within the sensory epithelium. Low-power confocal and epi-fluorescent images (Zeiss)
521 of the hair cell layer were used to reconstruct the entire cochlear sensory epithelium. The
522 resulting composite images were used to count ectopic inner hair cells, measure the total length
523 of the sensory epithelia and used to define basal, mid and apical segments (~1400 μm). The
524 apical tip (~300 - 500 μm) was excluded from the analysis. For hair cell and supporting cell
525 counts a series of high-power confocal (Zeiss) z-stack images spanning the hair cell and
526 supporting cell layer were taken within the basal, mid and apical segments. Images were
527 assembled and analyzed in Photoshop CS6 (Adobe). Image J software (National Institutes of
528 Health) was used to measure the length of counted segments and total length of the sensory
529 epithelium.

530

531 **Proliferation assay.** EdU (5-ethynyl-2'-deoxyuridine, Invitrogen) was reconstituted in PBS and
532 administered at 50 μg per gram of body weight to time-mated pregnant dams per intraperitoneal
533 injection. Click-iT AlexaFluor-488 or -546 Kit (Invitrogen) was used to detect incorporated EdU
534 according to the manufacturer's specifications. To quantify the number and percentage of EdU
535 positive hair cells and supporting cells, EdU stained cochlear surface preparations were co-
536 stained with the nuclear dye Hoechst-33258 (Sigma) and immuno-stained for SOX2, and
537 Myo7a. Length measurement and cell counts were conducted as described above.

538

539 **Statistical reporting.** Values are presented as mean \pm standard error of the mean (SEM). The
540 sample size (n) represents the number of biological independent samples (biological replicates)
541 analyzed per experimental group. Two-tailed unpaired Student's tests were used to determine
542 the confidence interval p-values ≤ 0.05 were considered significant. P-values > 0.05 were
543 considered not significant. Biological independent samples (biological replicates) were allocated

544 into experimental groups based on genotype and/or type of treatment. A minimum of three
545 biological independent samples were analyzed per group. To avoid bias masking was used
546 during data analysis.

547
548

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557

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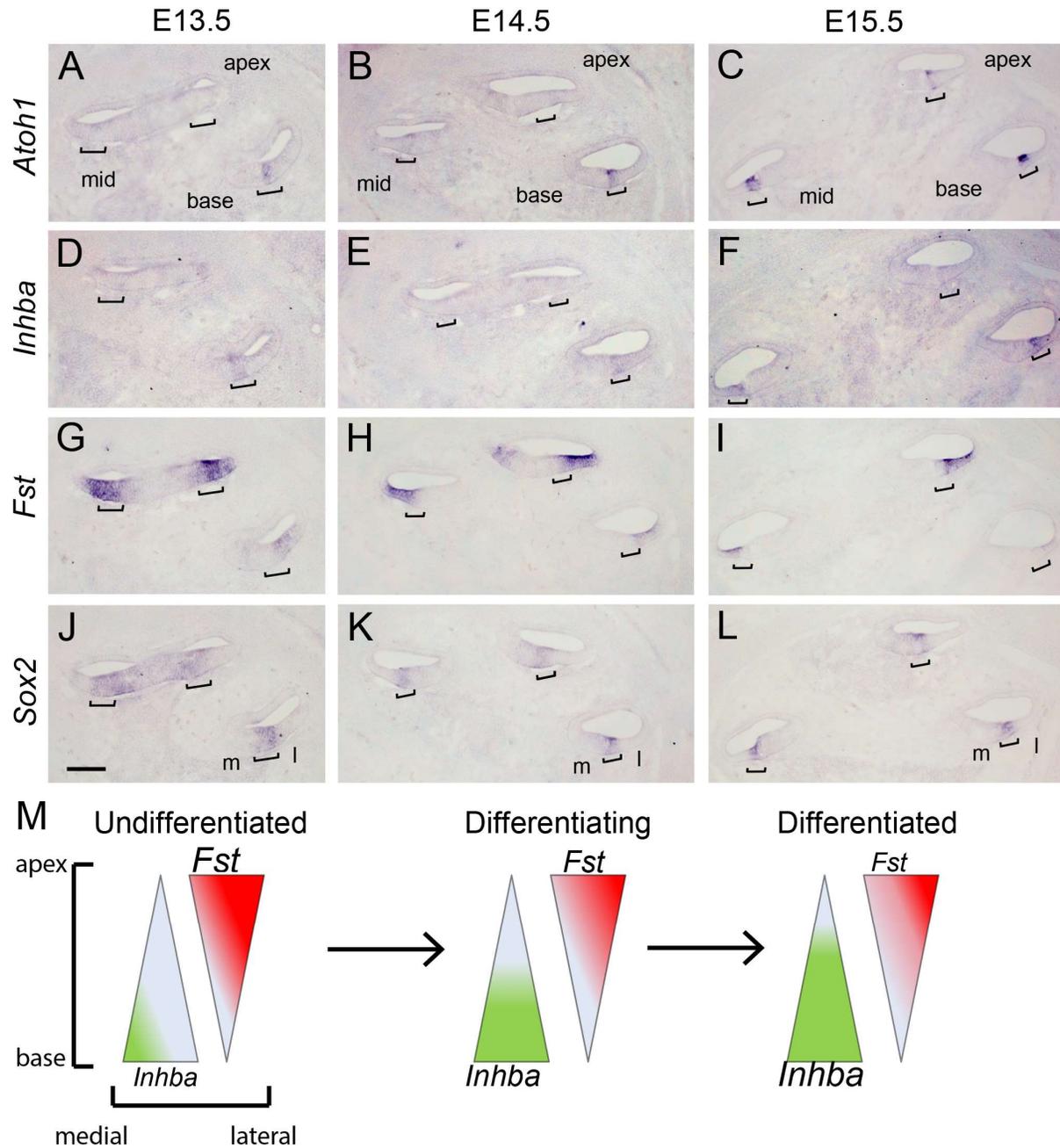
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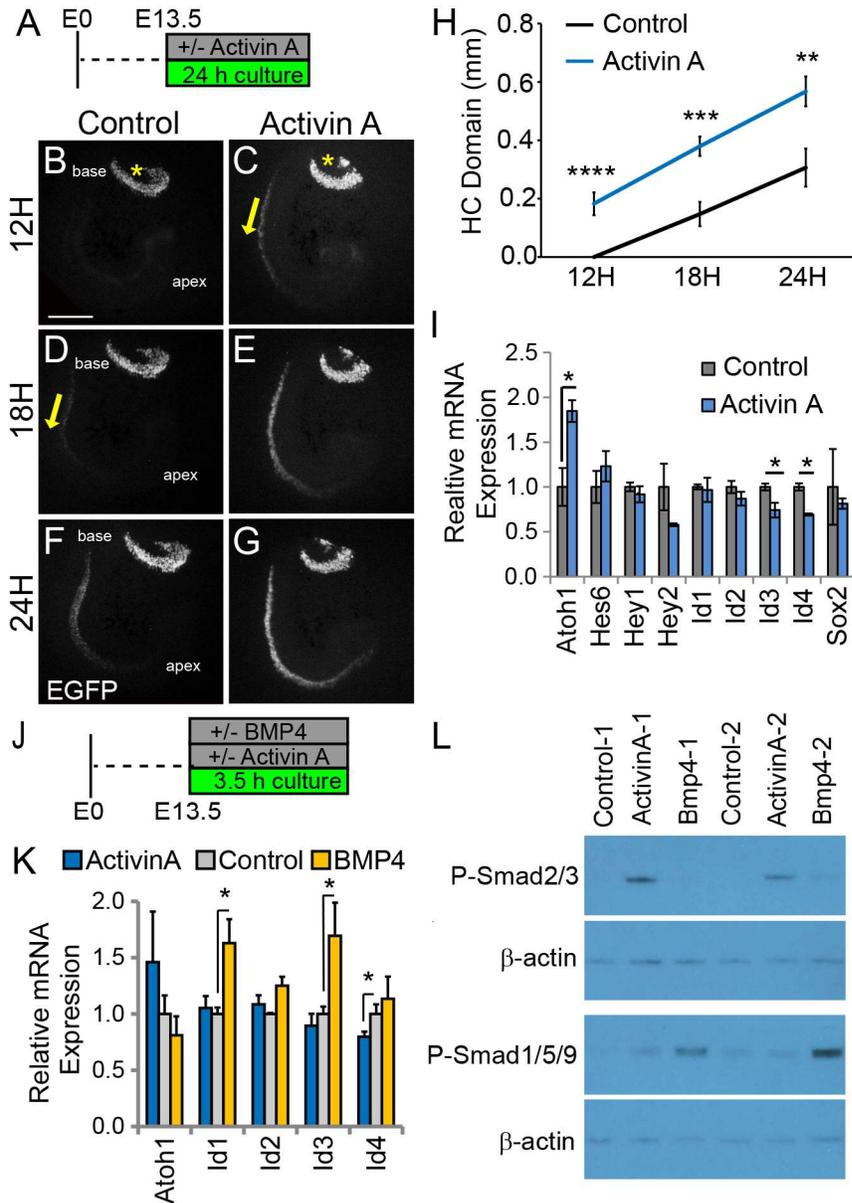
698 **FIGURE LEGENDS**



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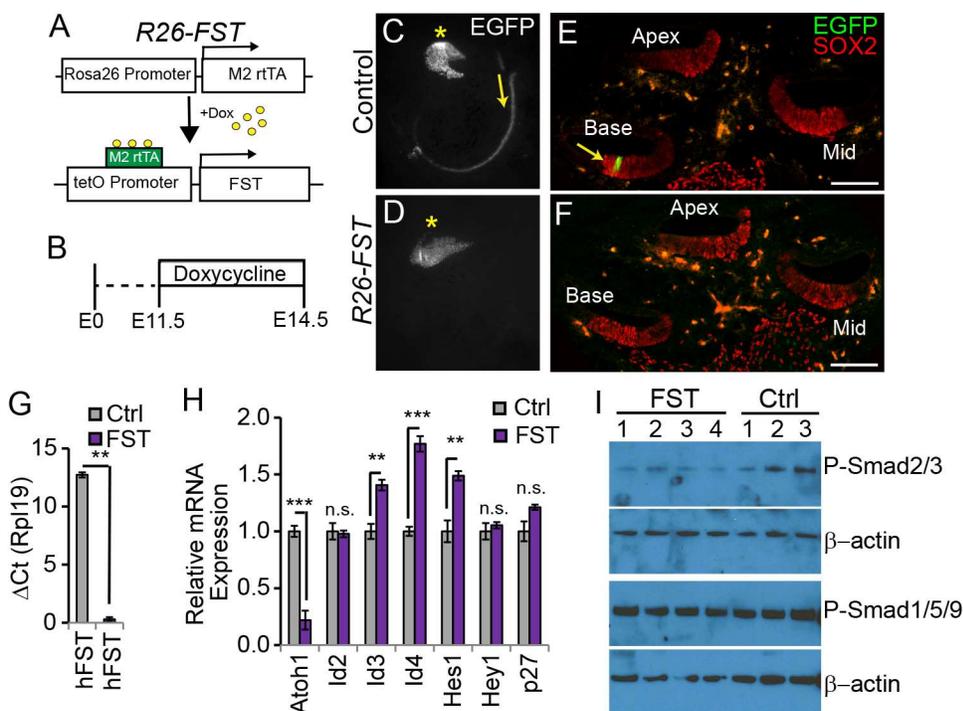
700 **Figure 1: Activin A expression parallels auditory hair cell differentiation. A-L:** In situ
701 hybridization (ISH) was used to analyze the cochlear expression pattern of *Inhba* (D, E, F) and
702 *Fst* (G, H, I) at the onset (E13.5) and during hair cell differentiation (E14.5 and E15.5). *Sox2* (J,
703 K, L) expression was used to mark the pro-sensory/sensory domains, *Atoh1* (A, B, C) marks

704 nascent hair cells. Brackets mark the pro-sensory/sensory domains with the cochlear duct.
 705 Abbreviations: m, medial; l, lateral. Scale bar, 100 μ m. **M**: Schematics of longitudinal (apical–
 706 basal) and radial (medial-lateral) expression gradients of *Inhba* and *Fst* within the auditory
 707 sensory epithelium.



708
 709 **Figure 2: Activin A promotes auditory hair cell differentiation.** **A**: Experimental design for
 710 **B-I**. Stage E13.5 wild type cochlear explants were cultured with or without Activin A (final conc.
 711 500 ng/ml) for 24 hours. **B-G** Atoh1/nEGFP reporter expression (EGFP, gray) was used to

712 monitor and analyze hair cell differentiation in Activin treated (C, E, G) and control (B, D, F)
713 cochlear explants. Asterisks marks vestibular sacculus that contains Atoh1-positive hair cells.
714 Yellow arrows mark the onset of hair cell differentiation within the cochlea. Scale bar, 100 μ m.
715 **H:** Quantification of extent of hair cell differentiation in control versus Activin treated cochlear
716 cultures (see B-G). Data expressed as mean \pm SEM (n = 5-8 cochlear explants per group, **p <
717 0.01, ***p < 0.001, ****p < 0.0001). **I:** Transcript levels of pro-sensory genes (*Id1-4*, *Hey1*, *Hey2*
718 and *Sox2*) and hair cell-specific genes (*Atoh1*, *Hes6*) were analyzed in enzymatically purified
719 cochlear epithelia. 3-4 cochlear epithelia were pooled per sample. Data are mean \pm SEM (n=3
720 independent experiments, *p < 0.05). **J:** Experimental design for K, L. Stage E13.5 wild type
721 cochlear epithelia were cultured with or without Activin A (final conc. 200 ng/ml) or BMP4 (final
722 conc. 100ng/ml) for 3.5 hours. **K:** RT-qPCR analysis reveals differential response to Activin and
723 BMP treatment. Individual cochlear epithelia were analyzed. Data are mean \pm SEM, n=4
724 biological replicates, *p < 0.05. **L:** Activin A induces Smad2/3 phosphorylation in cochlear
725 epithelial cells. Western blot analysis was used to establish P-Smad2/3 and P-Smad1/5/9
726 protein levels in individual cochlear epithelial extracts after 3.5 hour exposure to Activin A or
727 BMP4. Beta-actin was used as loading control.



728

729 **Figure 3: FST inhibits auditory hair cell differentiation.** **A:** Inducible FST transgenic mouse

730 model. In the presence of doxycycline (dox) double transgenic animals (R26-M2rtTA and tetO-

731 hFST) express human FST under the control of the R26 promoter (R26-FST). Non-transgenic

732 littermates and littermates that carry only one of the transgenes were used as experimental

733 controls (Ctrl). **B:** Experimental strategy for C-H. Timed mated pregnant dams received dox at

734 stage E11.5 and FST transgenic (R26-FST) animals and control littermates (Ctrl) were

735 harvested at E14.5. **C, D:** Low power fluorescent images of native Atoh1/nEGFP reporter

736 expression (EGFP, grey) of FST overexpressing (D) and control cochlear epithelia. Scale bar

737 100 μ m. **E, F:** Confocal images FST overexpressing (F) and control cochlear cross sections (E).

738 Native Atoh1/nEGFP (EGFP, green) marks hair cells (yellow arrow); SOX2 staining (red) marks

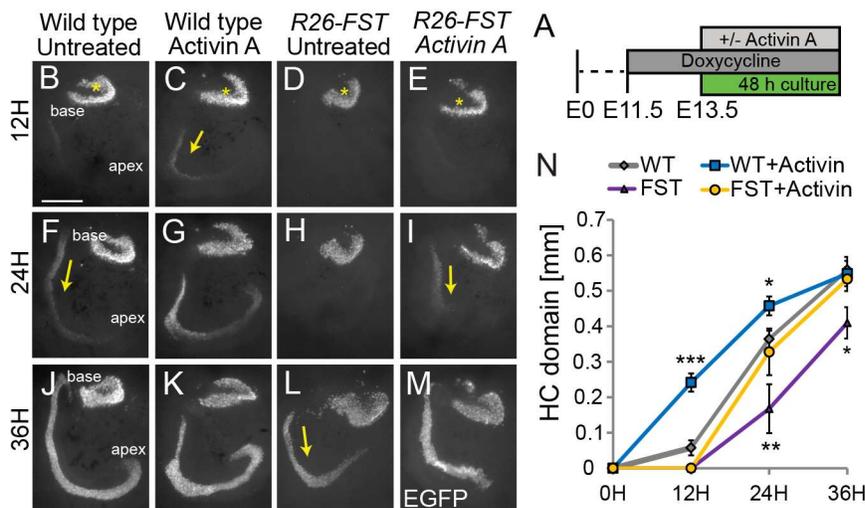
739 the sensory domain. Scale bar 100 μ m. **G:** Human (h) *FST* transgene expression in control

740 (Ctrl) and *R26-FST* transgenic (FST) cochlear epithelia. Plotted is their relative quantity (Δ Ct)

741 compared to the reference gene Rpl19. Data expressed as mean \pm SEM (n = 4-5 animals per

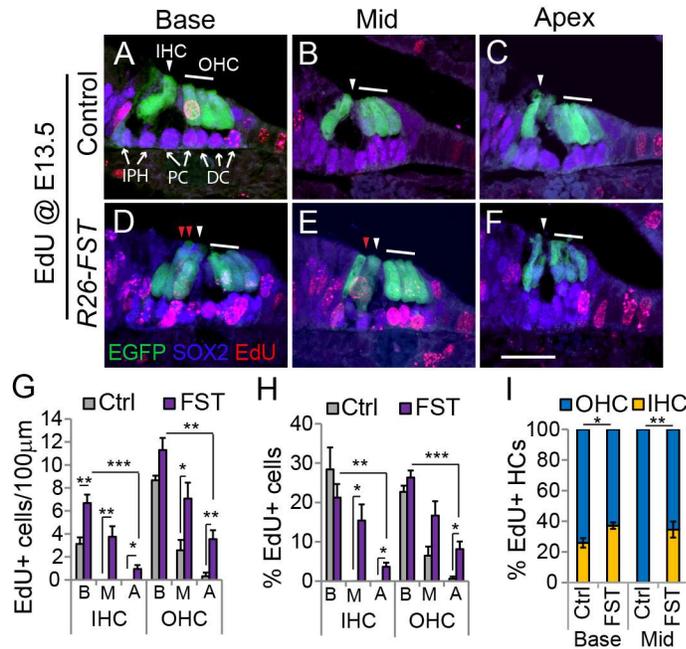
742 group, **p \leq 0.01). **H:** RT-qPCR-based analysis of gene expression in FST overexpressing (FST)

743 and control cochlear epithelia (Ctrl). Data are mean \pm SEM ($n = 4-5$ animals per group, * $p <$
 744 0.05 , ** $p < 0.01$, *** $p < 0.001$). **I:** Western-blot based analysis of P-SMAD2/3 and P-SMAD1/5/9
 745 protein levels in *FST* overexpressing (R26-FST: 1-4) and control (control: 1-3) cochlear
 746 epithelia. Timed mated pregnant dams received dox at starting at E12.5 and cochlear epithelia
 747 were isolated two days later from stage E14.5 *FST* transgenic (R26-FST) animals and control
 748 littermates (Ctrl). Beta-actin was used as loading control.



749 **Figure 4: Exogenous Activin A rescues the FST induced delay in auditory hair cell**
 750 **differentiation. A:** Experimental design for B-N. Dox was administered to timed pregnant dams
 751 starting at E11.5. At E13.5, cochlear tissue from *FST* overexpressing embryos and control
 752 littermates were cultured for 48 hours with or without Activin A (500 ng/ml). **B-M:** Atoh1/nEGFP
 753 reporter expression (EGFP, grey) marks nascent hair cells. Asterisks indicate hair cells within
 754 vestibular sacculus. Yellow arrows mark nascent cochlear hair cells. Scale bar, 100 μ m. **N:**
 755 Length of Atoh1/nEGFP positive sensory epithelium was used to quantify the extent of hair cell
 756 differentiation in control and *FST* overexpressing cochlear explants cultured with and without
 757 Activin A. Data expressed as mean \pm SEM ($n = 5-18$ cochlear explants per group, $p \leq 0.05$, ** p
 758 < 0.01 , *** $p < 0.001$). Two independent experiments were conducted.

760



761

762 **Figure 5: FST overexpression delays pro-sensory cell cycle exit.** To induce *FST* transgene

763 expression, timed mated pregnant dames received dox beginning at E11.5. A single EdU pulse

764 was administered at E13.5 and EdU incorporation (red) in hair cells and supporting cells was

765 analyzed at stage E18.5. **A-F:** Confocal images of cross-sections through the base, mid and

766 apical turn of control (A-C) and FST overexpressing (D-F) cochlear tissue. *Atoh1/nEGFP*

767 transgene expression (green) marks inner hair cells (IHC, white arrowhead) and outer hair cells

768 (OHC, white bar). *SOX2* immunostaining (magenta) marks supporting cells including inner

769 phalangeal cells (IPH), pillar cells (PC) and Deiters' cells (DC) marked by white arrows. Ectopic

770 inner hair cells are marked by red arrowheads. Scale bar, 50 µm. **G-I:** Quantification of EdU

771 positive hair cells within the base, mid and apex of control (Ctrl, grey bars) and FST

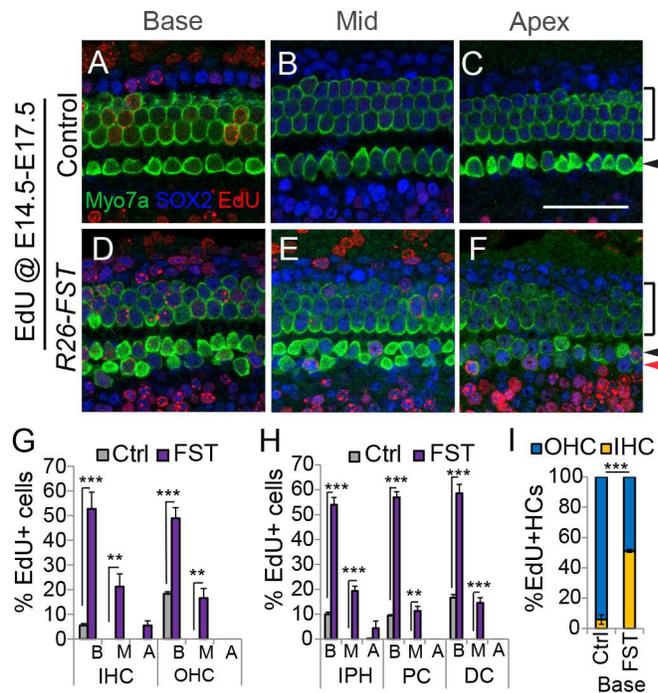
772 overexpressing (FST, purple bars) cochlear whole mounts. Graphed are the number of EdU

773 positive inner and outer hair cells per 100 µm (G), the percentage of inner and outer hair cells

774 that are EdU positive (H) and the percentage of EdU positive hair cells that are inner versus

775 outer hair cells (I). Abbreviations: IHC, inner hair cells; OHC, outer hair cells; B, base; M, mid; A,

776 apex. Data expressed as mean \pm SEM (n =4 animals per group, *p \leq 0.05, **p < 0.01, ***p <
 777 0.001).



778

779 **Figure 6: FST overexpression increases inner hair cell progenitor proliferation.** To induce

780 *FST* transgene expression, timed mated pregnant dames received dox beginning at E11.5.

781 Timed mated pregnant dams received two pulses of EdU daily starting from E14.5 to E17.5 and

782 EdU incorporation (red) in hair cells and supporting cells was analyzed at stage E18.5. **A-F:**

783 Confocal images of representative fields of basal, mid and apical segments of control (A-C) and

784 *FST* overexpressing (D-F) auditory sensory epithelia. Myo7a immuno-staining (green) marks

785 inner (black arrowhead), outer (black bar) and ectopic inner hair cells (red arrow head). Nuclear

786 SOX2 immuno-staining (blue) marks surrounding supporting cells and less mature hair cells.

787 Scale bar, 50 μ m. **G-I:** Quantification of EdU positive hair cells and supporting cells within the

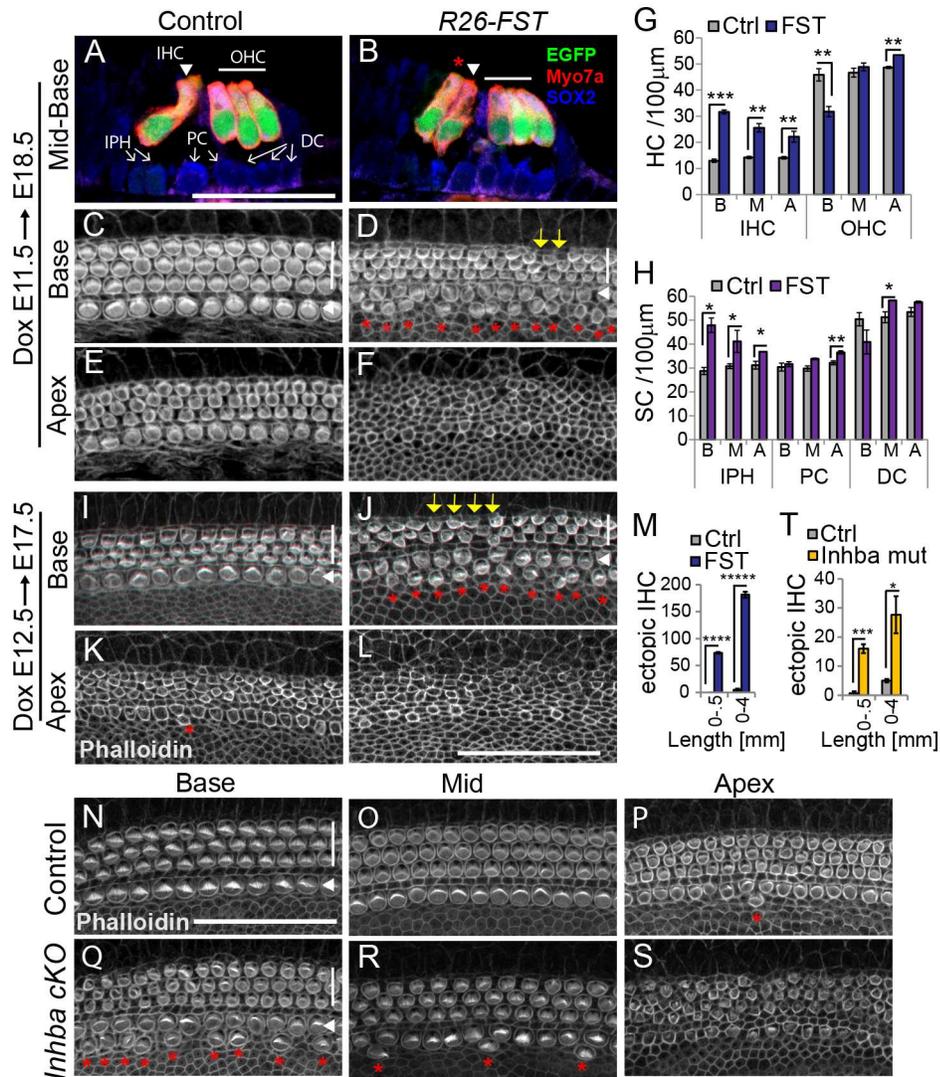
788 base, mid and apex of control (Ctrl, grey bars) and *FST* overexpressing (*FST*, purple bars)

789 cochlear whole mounts. Graphed are the percentage of hair cell (G) and supporting cell sub-

790 types (H) that are EdU positive and the percentage of EdU positive hair cells that are inner

791 versus outer hair cells (I). Abbreviations: IHC, inner hair cells; OHC, outer hair cells; IPH, inner

792 phalangeal cells; PC, pillar cell; DC, Deiters' cell; B, base; M, mid; A, apex. Data expressed as
 793 mean \pm SEM (n = 3 animals per group *p \leq 0.05, **p < 0.01, ***p < 0.001).



794
 795 **Figure 7: Disruption of Activin signaling increases inner hair cell formation and delays**
 796 **hair cell differentiation. A-T:** FST transgenic (R26-FST) embryos and their control (wild type
 797 or single transgenic) littermates were exposed to dox starting at E11.5 until tissue harvest at
 798 E18.5 (A-H) or starting at E11.5 until tissue harvest at E17.5 (I-T). **A-B: FST overexpression**
 799 **results in ectopic inner hair cell formation.** Confocal images of cross-sections through the
 800 midbase of control (A) and *FST* overexpressing (B) cochleae. *Atoh1/nEGFP* (green) and *Myo7a*
 801 (red) labels inner (white arrowhead), outer (white bar) and ectopic inner hair cells (red asterisk).

802 SOX2 (blue) labels supporting cells including inner phalangeal cells (IPH), pillar cells (PC) and
803 Deiters' cells (DC) indicated by white arrows. Scale bar 50 μ m. **C-L: FST overexpression**
804 **delays hair cell differentiation/maturation.** Confocal images of the apical surface of hair cells
805 located at the base (C, D, I, J) and apex (E, F, K, L) of *FST* over-expressing (D, F, J, L) and
806 control (C, E, I, K) cochlear whole mount preparations. Phalloidin labels actin-rich stereocilia of
807 inner (white arrowhead) and outer hair cells (white bar). Red asterisks mark ectopic inner hair
808 cells. Yellow arrows mark location of missing outer hair cells. Scale bar 50 μ m **G-H:**
809 Quantification of hair cells (G) and supporting cell (H) density in the base, mid and apex of
810 control (Ctrl, grey bars) and *FST* overexpressing (*FST*, purple bars) cochleae. Abbreviations:
811 IHC, inner hair cells; OHC, outer hair cells; IPH, inner phalangeal cells; PC, pillar cell; DC;
812 Deiters' cell, B, base, M, mid, A, apex. Data expressed as mean \pm SEM (n = 3 animals per
813 group, *p \leq 0.05, **p < 0.01, ***p < 0.001). **M:** Graphed are the number of ectopic inner hair cells
814 (IHC) within the most basal segment (0-.5 mm) and within the entire length (0 - 4mm) of control
815 (Ctrl, grey) and *FST* overexpressing (*FST*, purple) cochleae. Data expressed as mean \pm SEM (n
816 = 3 animals per group, ****p < 0.0001, ***** p < 0.00001). **N-T: Loss of *Inhba* delays hair cell**
817 **maturation and causes a mild overproduction of inner hair cells.** Shown are confocal
818 images of the apical surface of hair cells located in the base (N, Q), mid (O, R) and apex (P, S)
819 of stage P0 *Inhba* mutant (*Inhba* cKO) (Q-S) and control (Ctrl) cochlear whole mount
820 preparations (N-P). Phalloidin labels actin-rich stereocilia of inner (white arrowhead) and outer
821 hair cells (white bar). Red asterisks mark ectopic inner hair cells. Scale bar 50 μ m. **T:** Graphed
822 are the number of ectopic inner hair cells (IHC) within the most basal segment (0 - .5 mm) and
823 within the entire length (0 - 4mm) of control (Ctrl, grey) and *Inhba* mutant (*Inhba* mut, orange)
824 cochleae. Data expressed as mean \pm SEM (n = 3 animals per group, *p \leq 0.05, ***p < 0.001).
825
826