Targeted Deletion of All Known Thyroid Hormone Receptors Causes Maturation Retardation and Early-onset Degeneration of Cochlear Outer Sulcus

Xian-Hua Ma[1#], Fei Jiang[2#], Shuang Han[3], Yuqing Zhang[1,2], Lianhua Sun[2], Jiaxi Qu[4], Hao Yin[5], Yuxia Chen[1], Jie Tang[4], David Z. He[6], Weiping J. Zhang[1,3,*], Zhifang Xie[2,*]

[1]Department of Pathophysiology, Naval Medical University, Shanghai 200433, China
[2]Ministry of Education-Shanghai Key Laboratory of Children's Environmental Health, Institute of Early Life Health, Xinhua Hospital, Shanghai Jiaotong University School of Medicine, Shanghai 200092, China
[3]NHC Key Laboratory of Hormones and Development, Chu Hsien-I Memorial Hospital & Tianjin Institute of Endocrinology, Tianjin Medical University, Tianjin 300134, China
[4]Department of Physiology, Southern Medical University, Guangzhou, Guangdong 510515, China
[5]CAS Key Laboratory of Nutrition, Metabolism and Food Safety, Shanghai Institute of Nutrition and Health, University of Chinese Academy of Sciences, Chinese Academy of Sciences, and Shanghai Jiao Tong University Affiliated Sixth People's Hospital, Shanghai 200031, China.
[6]Department of Biomedical Sciences, Creighton University School of Medicine, Omaha, NE 68178, USA

[#]They contributed equally to the work.

***Corresponding authors**: Zhifang Xie (zfxie70@hotmail.com); Weiping Zhang, zbtb20@aliyun.com;

Author Contributions: ZX and WZ conceived the idea, and wrote the manuscript; ZX, WZ, DH and JT analyzed data and revised the manuscript; HY provided animal models; XM, FJ, JT, LS, YC, and SH performed most of the experiments. All authors were involved in final approval of the submission of the manuscript.

Competing Interest Statement: All the authors claim there is no interest of conflict about this work.

Classification: Developmental biology, Neuroscience

Keywords: Cochlea, Thyroid hormone receptor, development, root cell, transcription factor

Abstract

Thyroid hormone (TH) and/or its receptors (TRs) are essential for hearing, cochlear late-stage development and maintaining endocochlear potential (EP). The mechanism underlying the EP reduction upon deficiency of TH or TRs remains unknown. The cochlear outer sulcus (root cell) is considered to be important for the maintenance of endolymph homeostasis and EP, and undergoes dramatic morphological changes during cochlear late-stage developmental window. It has been long neglected whether TH and/or TRs are required for root cell differentiation and function. Here we demonstrate a key role for TH or TRs in postnatal root cell development and survival in mice. Anti-thyroid drug-induced developmental hypothyroidism results in severe retardation in root cell differentiation. Targeted deletion of both TRα and TRβ but not individual TRβ in cochlear epithelium causes a similar impairment, compounded with an early-onset degeneration of root cells and a reduced EP, without obvious alterations in stria vascularis. Furthermore, we demonstrate that TH signaling acts in parallel with ZBTB20 in the outer sulcus, a transcription factor which we have recently reported to be essential for the outer sulcus differentiation. Our results support that TRα and TRβ act redundantly in promoting root cell late-stage differentiation and maintaining its cellular homeostasis, and root cell dysfunction at least partially contribute to a reduced EP upon deficiency of TH or TRs. These findings imply that root cells may play an important role in maintaining the health of cochlear sensory epithelium and normal EP.

Significance Statement

Thyroid hormone (TH) and/or its receptors (TRs) are essential for hearing and cochlear development. The cochlear outer sulcus (root cell) is considered to be important for cochlear ionic and metabolic homeostasis. We found that combined deletion of both TRα and TRβ but not individual TRβ results in severe retardation in root cell differentiation accompanied by an early-onset degeneration and a reduced endocochlear potential (EP), suggesting TRα and TRβ act redundantly in promoting root cell differentiation and maintaining its cellular homeostasis. Our findings suggest that root cell dysfunction may at least partially contribute to a reduced EP upon deficiency of TRs, and root cells may play an important role in maintaining the health of cochlear sensory epithelium and EP.

Introduction

Thyroid hormone (TH) is necessary for normal development of cochlea and of hearing(1). Both early-onset congenital hypothyroidism and environmentally based iodine deficiency can cause hearing loss in humans and rodents(2–4). TH exerts its function mainly by binding to two different thyroid hormone receptors (TRs), namely TRα and TRβ, which are ligand-dependent transcription factors encoded by *Thra* and *Thrb*, respectively (5–7). TRα is individually dispensable for hearing(8), while lack of TRβ alone causes deafness accompanied by tectorial membrane (TM) malformation (5, 7). Deficiency of all known TRs by combined global deletion of *Thra* and *Thrb* in mice results in exacerbated cochlear defects including a more severe distorted TM, a severe retardation in the regression of great epithelial ridge (GER, also known as Kolliker's organ) and remodeling of the organ of Corti, as well as a reduced endocochlear potential (EP) (6). The mechanism underlying EP reduction upon TRs deficiency remains enigmatic, as no obvious stria vascularis (SV) alterations have been reported in these mice, indicating defects beyond SV might contributing to the EP reduction.

Cochlear outer sulcus epithelial cells (root cells) are poorly understood cell types that are considered to be important for the maintenance of endolymph homeostasis and EP (9–13). The outer sulcus is located on cochlear lateral wall between the outer boarder Claudius cells and the spiral prominence, and undergoes dramatic morphological changes during postnatal developmental window, when the cochlea displays the greatest sensitivity to TH (14–16). At birth, the outer sulcus is composed of a simple layer of cuboidal-shaped epithelial cells lining the scala media. Shortly after birth, these cells start to infiltrate laterally and superiorly into the spiral ligament underlying the spiral prominence and develop extensive tree-like branched

processes, and thus be designated as “root cells”. During the same period, Claudius cells become greatly enlarged and meet the spiral prominence epithelium covering the underlying root cell bodies, so that root cells are no longer directly in contact with the endolymph in basal turns. In the apical turn, however, root cells face the endolymph directly and have fewer processes(9, 11, 13, 17–19).

Little is known about how root cell differentiation is controlled. We have recently reported that transcription factor ZBTB20 is essential for cochlear late-stage development including root cell differentiation. Cochlear epithelium selective deficiency of ZBTB20 causes deafness, EP reduction and cochlear malformation including a deformed TM and the organ of Corti , as well as a failure in the maturation of the outer border and outer sulcus region (20). Very interestingly, *Thrb* is expressed by the developing and adult root cells as revealed by using a *lacz* gene reporter(21) or single-cell RNA sequencing (22), and *Thra* mRNA is widely expressed by the lateral wall including root cells (23, 24). It remains unknown whether TH and the expression of TRs in cochlea are essential for remodeling and maturation of the outer sulcus.

Here we find that TRs expression in otocyst-derived cochlear epithelium is necessary for postnatal root cell development and survival. We show that root cell development was severely retarded in antithyroid drug-induced developmental hypothyroidism or otocyst-selective *Thra* and *Thrb* double knockout mice, while no obvious root cell defects were observed upon individual *Thrb* deletion. Absence of both TRα and TRβ also led to an early-onset degeneration of root cells and a compromised EP value. We further show that TH signaling acts in parallel with ZBTB20 in controlling outer sulcus development.

Results

Hypothyroidism results in the disruption in the outer sulcus development.

To determine the role of thyroid hormone in the outer sulcus development, developmental hypothyroidism was induced by antithyroid treatment as previously described (25). The antithyroid treatment was initiated on day 17 of gestation and continued during lactation until postnatal day (P) 21, which covers the developmental window when cochlea shows high sensitivity to TH deprivation (14, 26). We first performed histological analysis on the cochleae from eu- or hypothyroid mice at P21, when cochlea becomes mature morphologically and functionally in normal mice(18, 27). We applied a resin-embedding technique to better preserve cochlear histological features. The semi-thin sections of cochlear basal turns revealed that euthyroid cochlear sensory epithelium displayed a typical mature morphology: an upright organ of Corti, a well-developed inner sulcus and a normal TM. In the outer sulcus region, enlarged Claudius cells covered the underlying root cells, which showed numerous bright branched processes extending deep into the spiral ligament and upward to behind the spiral prominence (Fig. 1*A*). Consistent with previous report(14), the hypothyroid cochlear epithelium displayed a severe retardation in the sensory epithelium remodeling manifested by an abnormal persistence of GER, closure of tunnel of Corti, and a severe distorted TM. Interestingly, the outer sulcus also displayed an immature appearance: a simple layer of cells with a few short processes bordering the scala media, continued with the flattened Claudius cells, suggesting a severe retardation in the root cell and Claudius cell differentiation (Fig. 1*B*).

We and others have previously shown that root cell cytoplasm and their processes are enriched with acetyl-α-tubulin (13, 20, 28). To better visualize root cells, we then performed

126 immunofluorescence staining for acetyl-α-tubulin. Consistent with the above histological
127 observations, euthyroid cochleae displayed robust and extensive acetyl-α-tubulin signals deep
128 into the spiral ligament (Fig. 1*C*), whereas the hypothyroid counterparts only showed limited
129 signals distributed mainly around the luminal surface of the spiral ligament, suggesting root cell
130 differentiation defects (Fig. 1*D*). Thus, TH signaling is necessary for the timely development of
131 the outer sulcus

132 **Cochlea-selective deletion of all known TRs causes deafness and a compromised**
133 **EP.**

134 As TH effects are mainly mediated by TRα and TRβ(1), both of which are expressed in the
135 developing outer sulcus(21–23), we therefore asked if the two receptors are essential for root
136 cell differentiation. To answer this question, we generated mice with individual *Thrb* or combined
137 with *Thra* gene selectively deleted in otic vesicle (hereafter referred as OV-TRβKO and OV-
138 TRα/βKO, respectively) using the Cre-loxP system. Individual *Thra*-deleted mice were excluded
139 in this study as *Thra*-null mice have normal hearing threshold(8). We crossed *Thrb-floxed*
140 (*Thrb*^{flox}) mice(29) individually or combined with *Thra* ^{flox} mice(30) to *Foxg1-Cre* transgenic
141 mice, a line that can mediate recombination selectively in the otic vesicle by E8.5(31). We
142 confirmed the tissue specificity of Cre-mediated recombination by mating *Foxg1-Cre* mice with
143 Rosa26-lacZ reporter mice. Accordingly, Cre-recombinase activity (displayed by X-gal staining)
144 was confined to otocyst-derived cochlear epithelium at P5, including GER, less epithelial ridge
145 (LER), outer sulcus epithelium (root cells) and stria vascularis (SV) marginal cells, as well as
146 spiral ganglions. As expected, no signal was detected in cochlear fibrocytes which are derived
147 from the periotic mesenchyme surrounding the otocyst (32). No background signal was

148 detected on *cre*-negative control mice (Fig. 2*A*). The deletion efficiency was further determined
149 by real-time RT-PCR analysis showing that expression levels of *Thrb* but not *Thra* mRNA were
150 significantly down regulated in OV-TRβKO cochleae at P10, while both of *Thrb* and *Thra* mRNA
151 were significantly down regulated in OV-TRα/βKO cochleae (Fig. 2*B*).

152 To evaluate the auditory function of the mutant mice, we performed ABR (auditory
153 brainstem response) tests. Both of OV-TRβKO and OV-TRα/βKO mice did not show typical
154 ABR responses to either click（Fig. 2*C*, *D*）or frequency-specific tone burst stimuli at 90 dB
155 (the highest intensity tested) around 2 months（Fig. 2*D*), in sharp contrast to normal thresholds
156 seen in wild-type (WT) control mice. These data demonstrate that cochlear expression of TRβ
157 or all known TRs is essential for hearing, in agreement with previous reports(5, 6).

158 As global deletion of all known TRs results in a ~50% reduction in EP magnitude in adult
159 mice (6), we thus measure EP from OV-TRα/βKO mice. The OV-TRα/βKO mice showed a slight
160 but statistically significant reduction in EP magnitude when compared with WT controls at ~1.5
161 months of age（Fig. 2*E*）, suggesting expression of TRs in cochlear epithelium contributes to
162 EP maintenance at this age.

163 **Cochlea-selective deficiency of all known TRs retards root cell development.**

164 We next investigated cochlear morphological changes in TRs mutant mice, with an
165 emphasis on the development of outer sulcus region. At P10, 2 days prior to the onset of hearing
166 in mice, root cells with branched processes had already invaded deep into the spiral ligaments
167 in WT pups (Fig. 3*A*). Comparable root cell development was also observed in OV-TRβKO
168 counterparts (Fig. 3*B*). The outer sulcus in OV-TRα/βKO mice, however, was largely composed
169 of a single cuboidal epithelial layer bordering the endolymphatic space (Fig. 3*C*), suggesting a

delay in the root cell differentiation. Consistent with the histological observations, OV-TRα/βKO showed apparently less acetyl-α-tubulin positive cell processes within the spiral ligament compared with those in WT and OV-TRβKO counterparts (Fig. 3*D-F*). To further demonstrate root cell differentiation, we examined expression patterns of *Pendrin* (*Slc26a4*) and *Epyc* (*Epiphycan*), which are abundantly and selectively expressed by root cells(20, 22, 33).) Strong signals of *Pendrin* or *Epyc* mRNA could be detected deep inside the spiral ligament in both WT and OV-TRβKO cochleae, but in OV-TRα/βKO cochleae, expression domains of both molecules were adjacent to the luminal surface of the spiral ligament (Fig. 3*G-I*, and *SI Appendix* Fig. S1*A-C*), consistent with a delay in the root cell development. These data demonstrate that root cell development appeared to be normally initiated in OV-TRβKO but was delayed in OV-TRα/βKO pups.

By P21, comparable mature morphological features were observed between OV-TRβKO and WT outer sulcus, showing elaborate root cell branches extending deep into the inferior region of spiral ligament and upward to behind the spiral prominence in the basal turn (Fig. 3*J* and *K*). As previously described(17, 34), the surface of root cell bodies was covered by greatly enlarged Claudius cells and spiral prominence epithelium, thus no longer directly exposed to the endolymph at this stage. The double knockout mice, however, showed only an initial sign of root cell differentiation. Root cells with a few processes, were mainly bordering the scala media, separating the flattened Claudius cell and the spiral prominence epithelium, suggesting a severe retardation in the differentiation of root cells and Claudius cells (Fig. 3*L*). It should be noted that some cells with a pale-staining cytoplasm and nucleus could be occasionally detected in OV-TRα/βKO outer sulcus at this age, suggesting degenerative changes (Fig. 3*L*).

192 The defects were further demonstrated by reduced acetyl-α-tubulin positive root cell processes
193 in OV-TRα/βKO outer sulcus, compared with WT or OV-TRβKO counterparts (*SI Appendix* Fig.
194 S1*D-F*). By 1.5 months, the development of root cells and Claudius cells in OV-TRα/βKO had
195 progressed to some extent but still lagged behind the developmental status in WT controls (*SI*
196 *Appendix* Fig. S2*A-B*, *A'* and *B'*). Degenerative changes in root cells and cochlear outer border
197 cells (Boettcher's cells) were noticed in some OV-TRα/βKO samples (*SI Appendix* Fig. S2*C*
198 and *C'*).

199 Taken together, these data show that the root cell development appeared intact in OV-
200 TRβKO but was severely retarded in OV-TRα/βKO mice, suggesting that TRβ is individually
201 dispensable and TRs may play redundant roles in root cell development.

202 **Deficiency of all known TRs causes early-onset root cell degeneration.**

203 Although the degeneration of cochlear hair cells and supporting cells has been reported
204 previously in developmental hypothyroidism, and in mice with global deletion of TH transporters
205 (35, 36, 4), it remains unknown if root cells survival depends on TH signaling. As described
206 above, sporadic degenerative cells were occasionally detected in OV-TRα/βKO outer sulcus at
207 P21 (Fig. 3*L*), or ~1.5 months of age (*SI Appendix* Fig. S2*C* and *C'*). By 2 months, WT control
208 or OV-TRβKO outer sulcus showed no obvious degenerative changes, whereas OV-TRα/βKO
209 exhibited apparently reduced root cells with pale-staining processes and nuclei in both basal
210 and apical turns (Fig. 4*A-C*, *A'-C'*, and *SI Appendix* Fig. S3), suggesting progressive
211 degenerations. Concomitant with the root cell degeneration, variable degenerations of the
212 organ of Corti were observed in OV-TRα/βKO cochlea, resembling those described previously
213 in developmental hypothyroidism, or in mice with global deletion of TH transporters (4, 35, 36).

214 To further evaluate root cell viability, we then quantified the number of root cell's nucleus
215 at 2 months. We and others have previously reported that Sox10 is localized in the nucleus of
216 cochlear non-sensory cells including outer border, root cells, spiral prominence epithelium but
217 is excluded from fibrocytes in the lateral wall (20, 37). Therefore, by performing double immune-
218 staining, root cells could be easily distinguished as Sox10 and acetyl-α-tubulin (intensely
219 expressed in the root cell cytosol) double positive cells in the spiral ligament (Fig. 4*D*).
220 Consistent with the histological observations, the number of root cell's nucleus was significantly
221 decreased in OV-TRα/βKO but not in OV-TRβKO, compared to that in WT, supporting root cell
222 degeneration upon the absence of both TRs (Fig. 4*D-E*). Thus, TRs are necessary and also
223 function redundantly for root cell survival at this age.

224 **Stria vascularis remains intact upon deletion of all known TRs in cochlea.**

225 As OV-TRα/βKO showed a reduced EP compared with control mice (See Fig. 2*E*), we then
226 examined the structural and functional integrity of SV, which plays an essential role in
227 generating EP and maintaining K^+ homeostasis in the endolymph(38–40). No obvious
228 histological abnormality was observed in either OV-TRβKO or OV-TRα/βKO SV at P21 (Fig.
229 5*A*). The area of SV from both mice lines was comparable to that from WT (Fig. 5*B*).
230 Consistently, immuno-staining analysis showed that both groups showed normal expression
231 patterns of EP-generating ion-transport molecules including KCNJ10 (Kir4.1, a marker for
232 intermediate cells)(41, 42), Na^+/K^+-ATPase α(43), NKCC1(44) and KCNQ1 (Kv7.1)(45, 46)
233 (markers for marginal cells) (Fig. 5*C*). These data suggest that SV remains intact upon all TRs
234 deletion in cochlear epithelium. Indeed, potential primary defects can be excluded from SV
235 intermediate cells and basal cells in either OV-TRβKO or OV-TRα/βKO mice, as these two cell

236 types are not originated from the otic epithelium(47) and thus should display no Cre
237 recombination activity (see Fig. 2*A*).

238 As gap junctions are also essential for EP generation and hearing(48, 49). We then
239 analyzed the expression of Connexin26 (Cx26) and Cx30 proteins, which are the dominant
240 gap-junction channel-forming proteins expressed in cochlear epithelium(50). Comparable
241 expression patterns of Cx26 and Cx30 were observed between control and OV-TRα/βKO
242 cochleae at P21 (*SI Appendix* Fig. S4), suggesting gap junction system remains intact upon
243 TRs deficiency.

244 **TH signaling acts in parallel with ZBTB20 in the outer sulcus development.**

245 We have recently reported that transcription factor ZBTB20 is essential for root cell
246 differentiation and maturation in mice(20). We therefore asked whether TH acts upstream of
247 ZBTB20 in regulating root cell differentiation. To test if TH regulates ZBTB20 expression in the
248 outer sulcus, we compared ZBTB20 protein levels in the drug-induced hypothyroid cochlea with
249 that in euthyroid during postnatal development stage. Western blot showed that ZBTB20 protein
250 levels were significantly decreased in the extracts of hypothyroid cochleae at P4 compared with
251 those in euthyroid controls (Fig. 6*A*, and *SI Appendix* Fig. S5). Immunostaining revealed that
252 the decrease in ZBTB20 expression was mainly detected in the GER epithelial cells of
253 hypothyroid cochleae at P5, while its expression in the rest of the cochlear epithelium including
254 supporting cells and the outer sulcus/root cells was comparable with those in euthyroid controls
255 (Fig. 6*B-D*). Consistently, comparable ZBTB20 expression was observed between OV-
256 TRα/βKO and WT lateral wall at P10 (*SI Appendix* Fig. S6). These data suggest that ZBTB20
257 expression in outer sulcus/root cells is not TH-dependent although its expression in the GER

258 may be regulated by TH.

259 We then tested if ZBTB20, instead, functions upstream of TH signaling pathway. OV-
260 ZB20KO mice had normal serum levels of tri-iodothyronine (T3), and thyroxine (T4) at P10 (*SI*
261 *Appendix* Fig. S7*A-B*). Quantitative RT-PCR analyses showed that OV-ZB20KO cochleae
262 expressed comparable mRNA levels of thyroid hormone transporters at P3 (*SI Appendix* Fig.
263 S7*C*), as well as TH receptors including *TRα1*, *TRβ1* and *TRβ2* at P10 (*SI Appendix* Fig. S7*D*).
264 In situ hybridization of cochleae revealed no significant difference in the mRNA expression
265 pattern of genes encoding type 2 and type3 deiodinases (*Dio2* and *Dio3*) (*SI Appendix* Fig.
266 S7*E*), or *TRα1* and *TRβ1* between the two genotypes (*SI Appendix* Fig. S7*F*). Thus ZBTB20
267 unlike acts upstream of TH signaling pathway.

268 Together, these results suggest that TH signaling may acts in parallel with ZBTB20 in
269 regulating the outer sulcus remodeling.

Discussion

This study demonstrates a key role for TH and TRs in postnatal root cell development and survival. Absence of both TRα and TRβ but not TRβ alone in cochlear epithelium resulted in severe retardation in root cell differentiation and an early-onset root cell degeneration, suggesting that TRα and TRβ act redundantly in promoting root cell late-stage differentiation and maitaining cellular homeostasis.

The critical period when TH is essential for cochlear development and normal hearing covers from late embryonic stages to around the second postnatal week in rodents(14, 26, 51). During this developmental window, cochlear sensory epithelium undergoes dramatic morphological changes including regression of GER leading to the formation of inner sulcus, and opening of the tunnel of Corti (14, 52). Concomitantly, the outer sulcus epithelium transformes from a simple layer of cuboidal-shaped cells lining the scala media into root cells with large, branched processes resided deep in the spiral ligament(18, 20, 52). This study uncovers that TH or TRs is nessesary for the outer sulcus remodeling, in addition to previous reported GER regression and the organ of Corti maturation(6). Although both TRα and TRβ are expressed by the developing outer sulcus(21–24), severe retardation in root cell differentiation was observed only in the absence of both receptors indicating that TRα and TRβ compensate functionally for each other in controlling root cell development. Our findings extend those data obtained from the mouse model with global deletion of TRs, which suggest synergistic effects of TRα and TRβ in controlling the remodeling of the organ of Corti and the correct formation of tectorial membrane (6).

Another intrigueing finding of this study is the early-onset progressive degeneration of root

cells observed only in OV-TRα/βKO but not OV-TRβKO cochlea, suggesting TRα and TRβ play a combined role in root cell survival. Interestingly, degeneration of root cells and the organ of Corti initiated concomitantly in juvenile OV-TRα/βKO cochlea, indicating potential coordinated mechanisms underlying these degenerations. As root cells with their extensive processes are closely associated with capillaries and fibrocytes within the spiral ligament, it has been sugested that root cells may provide a cellular route for transporting nutrients, blood-borne hormones (such as T3), metabolites and ions between the vasculature in the spiral ligament into the infeior cochlear sensory epithelium(13, 17, 19), which lacks direct blood flow. This hypothesis is supported by an epithelial gap junction network formed by the syncytium of supporting cells in the organ of Corti and extending through the Claudius' cells and root cells(53, 54). It is thus reasonable to assume that the proper timely developent of root cells may help to maintain the health of cochlear sensory epithelium. The early-onset degeneration of root cells might comprimise the nutrients and hormones supplement, or ion and metabolite homeostasis in the organ of Corti, promoting its degeneration. Alternatively, root cells health might be affected by the degeneration of organ of Corti. Severe early-onset degenerations of cochlear hair cells have been reported previously in congenital hypothyroidism, or in mice with global deletion of thyroid hormone transporters (4, 36), though the status of root cells in these animal models have not been reported. It would be worth to explore any alterations in root cells structure or function in these mice.

Several lines of evidence including our recently published report suggests that root cells may play important roles in the maintenance of endolymph homeostasis and EP(9, 12, 10, 20). Our findings in this study also support this hypothesis and imply that root cell

314 dysfunction may at least partially contribute to a reduced EP upon deficiency of TH or all
315 known TRs during the critical period of cochlear development (4, 6, 36).

316 The EP is generated and maintained mainly by the SV (38, 55–57). Although SV
317 defects have been reported in a secondary hypothyroidism caused by pituitary transcription
318 factor 1 (Pit1) mutant (4), no obvious SV alterations have been reported in either TH
319 transporters deficiency (36), or global *Thra* and *Thrb* double knockout mice(6), both of which
320 display a significant reduction in EP, indicating defects beyond SV might contributing to the EP
321 reduction in these mice models. Consistently, our findings also show a reduced EP without
322 obvious defects in SV upon deficiency of both TRα and TRβ in cochlear epithelium. Indeed,
323 *Thrb* mRNA is not detected in the developing SV, as revealed previously using a sensitive *LacZ*
324 gene reporter system(21).Thus the SV is unlike a primary defect site responsible for a reduced
325 EP, which occurred only in mice lacking all TRs(6). As mentioned above, root cells are
326 located at the lateral border of cochlear epithelial gap junction network adjacent to type II
327 fibrocytes and have been suggested a role in K^+ recycling(58). This hypothesis is supported by
328 a report showing deficiency of KCNK5 channel, which is mostly expressed by the outer sulcus,
329 results in profound deafness associated with a decreased EP(10). Consistently, our recent
330 report showed that disruption of outer sulcus development upon ZBTB20 deletion results in EP
331 reduction(20). Therefore, our results in this study are consistent with a role for TH and TRs in
332 the development and function of root cells.

333 EP reduction observed in juvenile OV-TRα/βKO mice appears to be less severe than that
334 reported in adult global *Thra* and *Thrb* double knockout mice(6). This discrepancy may be due
335 to that EP was recorded at a younger age in this study, as root cell degeneration may be

336 progressive, causing further decrease in EP magnitude. Alternatively, global deletion of both
337 *Thra* and *Thrb* may cause defects in other cell types such as fibrocytes in the lateral wall, which
338 also play a role in EP maintenance(59, 60). In OV-TRα/βKO mice, however, primary fibrocyte
339 defects can be excluded as the Cre recombination activity was confined in otocyst-derived
340 cochlear epithelium, as shown in Fig.1*A*. On the other hand, the average ~15 mV of EP
341 reduction might be an underestimate of the real change in EP in these mice. Previous studies
342 have shown that the magnitude of EP also depends on the status of mechanotransduction of
343 hair cells since mechanotransduction in the stereocilia acts like an adjustable resistor(61, 62).
344 Reduction in mechanotransduction and/or loss of hair cells can lead to an increase in EP
345 value(63, 64). The severely distorted TM in OV-TRα/βKO mice would cause the detachment of
346 the TM from stereocilia bundles of outer hair cells, resulting in more mechanotransduction
347 channels to be closed at rest, and thus increasing EP value to some extent. Therefore, the 15-
348 mV reduction of EP seen in these mice is the result of two opposite actions. It is thus highly
349 likely that the contribution of the outer sulcus to EP may be underestimated in juvenile OV-
350 TRα/βKO mice.

351 Finally, our results reveal that TH signaling regulates root cell differentiation mostly by
352 acting in parallel with ZBTB20, which we have recently demonstrated as an essential
353 transcription factor governing the late-stage differentiation of the outer sulcus region(20). The
354 root cell differentiation was permanently disrupted upon *Zbtb20* ablation, while was severely
355 retarded but had progressed to some extent around 1.5 months in OV-TRα/βKO mice (*SI*
356 *Appendix* Fig. S2). This evidence let us to propose that the proper development of the outer
357 sulcus requires a coordination between TRs and ZBTB20 or other transcriptional pathways.

358 **Materials and Methods**

359 **Animal models**

360 All animal maintenance and experiments were conducted in accordance with the guidelines
361 of the Naval Medical University Animal Ethics Committee. *Thra* ^{flox}(30), *Thrb*^{flox} (29), OV-
362 ZB20KO(20), or ROSA26-*LacZ* reporter (65)mice have been described previously. The OV-
363 TRβKO mice were generated by cross-breeding of *Thrb* ^{flox} mice with *Foxg1-Cre* mice (Jax,
364 Stock No. 004337)(66). The OV-TRα/βKO mice were then generated by cross-breeding of
365 heterozygous OV-TRβKO mice with *Thra* ^{flox} mice. All mice were maintained on a C57BL/6J
366 background. Floxed/*Cre*-negative littermate mice were served as wild-type controls. PCR
367 primers for genotyping are listed in *SI Appendix* Table S1. Hypothyroidism induction： Timed-
368 pregnant C57BL/6J mice were treated with either antithyroid agents (0.05% methimazole, 1.0%
369 sodium perchlorate monohydrate and 5.0% sucrose in drinking water)(25) or vehicle from
370 embryonic day 17. The treatment was continued during lactation and stopped until the day
371 indicated.

372 **Histological analysis**

373 Mice were transcardially perfused with 4% paraformaldehyde (pH 7.4). Dissected cochleae
374 were then perfused perilymphatically with 2.5% glutaraldehyde and fixed overnight, followed by
375 post-fixed with 1% osmium tetroxide. Cochleae were then decalcified, dehydrated and
376 embedded in Spurr's low-viscosity resin. Mid-modiolar sections (1 μm) were stained with 1.25 %
377 (w/v) toluidine blue. For quantitative analysis, the upper basal turns from three non-consecutive
378 sections were photographed at a 200x magnification and SV areas were measured using

379 Image-Pro Plus 6.0 program. A single cochlea from each animal and n ≥ 5 mice per genotype
380 were analyzed at a given age.

381 **Immunohistochemistry and X-Gal staining**

382 Immunostaining was performed essentially as previously described (20). X-gal staining was
383 done as previously published (67). The information for primary antibodies are listed in *SI*
384 *Appendix* Table S2. To quantify root cells, non-consecutive mid-modiolar sections per cochlea
385 were subjected to double immunostaining for Sox10 and acetyl-α-tubulin. The nuclei of double
386 positive cells within the outer sulcus region from at least 5 sections were counted manually,
387 blind to genotype.

388 For quantitative analysis of ZBTB20 immunostaining intensity, more than three non-
389 consecutive mid-modiolar paraffin-embedded sections for each cochlea (single ear was
390 analyzed for each animal) from hypothyroid and euthyroid mice at P5 were stained in parallel
391 with anti-ZBTB20 antibody. The images were acquired at X400 magnification by a camera
392 (DP80; Olympus) equipped with a fluorescence microscope (BX53; Olympus). All imaging
393 parameters were kept at the same for each group. The averaged staining intensities in nuclei
394 of cells from defined regions (greater epithelial ridge (GER), supporting cells (SC), outer sulcus
395 epithelium (OS) and hair cells (HC)) at the upper-basal turn were numerically quantified by the
396 ImageJ program. The mean intensities of each sub-region were divided by those of HC at the
397 same turn to get normalized mean intensities, as HCs did not express ZBTB20 at this stage
398 thus could serve as the internal negative control.

399 **Auditory brainstem response (ABR) test**

400 ABRs were recorded using a TDT RZ6/BioSigRZ system (Tucker-Davis Tech. Inc., US) as
401 previously described (20). Briefly, anesthetized mice were kept on a heated mat in a sound-
402 proof chamber. Click or pure tone stimuli from 4 to 32 kHz were generated. The sound intensity
403 was decreased from 90 to 10 dB sound pressure level (SPL) in 5 dB steps and auditory
404 thresholds were defined as the lowest sound intensity evoking reproducible and recognizable
405 waves.

406 **Endocochlear potential (EP) measurement**

407 EP recording through cochlear lateral wall was previously described (20, 68). The basal
408 turns from both ears were analyzed for each animal. In brief, mice were anesthetized and
409 tracheotomy was performed. The tympanic bulla was opened to display the cochlea. A small
410 hole was drilled in cochlear lateral wall of the basal turn, through which a micropipette electrode
411 filled with 150 mM KCl was advanced into the scala media using a micromanipulator. The
412 voltage changes during the insertion were continuously recorded using an amplifier
413 (MultiClamp 700B, Molecular Devices) combined with a digitizer (Digidata 1440A, Molecular
414 Devices).

415 **In situ hybridization.**

416 In situ hybridization was performed as described(20). In brief, mice were transcardially
417 perfused with 4% paraformaldehyde (pH 9.0) and cochleae were dissected and perfused
418 perilymphatically with the same fixative. The fixed and decalcified cochleae were cryostat
419 sectioned (14 μm thickness). Sections were then subjected to in situ hybridization as described
420 previously(69). Information of antisense cRNA probes are listed in *SI Appendix* Table S3.
421 Cochleae from n ≥ 4 mice per genotype at a given age were analyzed.

422 **Real-time RT-PCR**

423 Total RNA extraction, cDNA synthesis and quantitative Real-time PCR was performed as
424 described previously(20). Data were normalized to 36B4 (Rplp0, internal control) and presented
425 as fold changes of gene expression in the mutant mice compared to the WT Control. Each
426 sample was analyzed in duplicate. The primers used are shown in *SI Appendix* Table S1.

427 **Measurement of thyroid hormones.**

428 Blood samples were collected and separated into serum aliquots by centrifugation and
429 stored at −80ºC until assayed. Serum levels of T3 and T4 were measured using T3 ELISA kit
430 from MLBIO (Cat. #m1559442) and T4 CLIA kit from cloud-clone Corp. (Cat. #CCA452Ge),
431 according to the manufacturers' instructions.

432 **Western blot**

433 Total proteins of cochleae from 2 ears per animal were electrophoretically separated on 4–
434 15% gradient SDS–PAGE gels and transferred onto nitrocellulose membranes. Membranes
435 were probed with the following primary antibodies: anti-Zbtb20 monoclonal antibody clone 9A10
436 (1:1000, home-made)(67); anti-histione H3 (1:5,000, Cell Signaling Technology). Secondary
437 antibodies were either HRP-conjugated anti-rabbit or anti-mouse IgG (1:8,000, Vector
438 Laboratories). Signals were then developed using a Chemiluminescent Detection Kit (Thermo
439 Fisher, #34580). The blot images were acquired by Luminescent Image Analyzer (Las 4000,
440 GE Healthcare) and the densities of ZBTB20 bands were quantified and normalized to those of
441 internal control.

442 **Statistical analysis**

All statistical analysis was performed using GraphPad Prism 7 software. Values are shown as means ±SEMs. The data normality tests were first carried out, and then a two-tailed Student's t test and one-way or two-way ANOVA were used to compare the means between two groups and among three groups, respectively. $p \leq 0.05$ was considered statistically significant. The non-significant difference between samples is indicated as "ns"; p values of significant differences are shown in the graphs.

Data availability: Data supporting the findings of this study are available within the article and its Supplementary information files.

Acknowledgements. This work was supported by grants from the National Key R&D Program (2019YFA0802500, 2018YFA0800602), National Natural Science Foundation of China (32271162), and Collaborative Innovation Program of Shanghai Municipal Health Commission (2020CXJQ01).

455 **Reference**

456 1. L. Ng, M. W. Kelley, D. Forrest, Making sense with thyroid hormone--the role of T(3) in
457 auditory development. *Nat Rev Endocrinol* **9**, 296–307 (2013).
458 2. S. Christ, *et al.*, Hearing loss in athyroid pax8 knockout mice and effects of thyroxine
459 substitution. *Audiol Neurootol* **9**, 88–106 (2004).
460 3. M. S. Deol, Congenital deafness and hypothyroidism. *Lancet* **2**, 105–6 (1973).
461 4. M. Mustapha, *et al.*, Deafness and Permanently Reduced Potassium Channel Gene
462 Expression and Function in Hypothyroid Pit1dw Mutants. *The Journal of Neuroscience*
463 **29**, 1212 (2009).
464 5. D. Forrest, L. C. Erway, L. Ng, R. Altschuler, T. Curran, Thyroid hormone receptor beta is
465 essential for development of auditory function. *Nat Genet* **13**, 354–7 (1996).
466 6. A. Rusch, *et al.*, Retardation of cochlear maturation and impaired hair cell function
467 caused by deletion of all known thyroid hormone receptors. *J Neurosci* **21**, 9792–9800
468 (2001).
469 7. H. Winter, *et al.*, Deafness in TRbeta mutants is caused by malformation of the tectorial
470 membrane. *J Neurosci* **29**, 2581–2587 (2009).
471 8. A. Rüsch, L. C. Erway, D. Oliver, B. Vennström, D. Forrest, Thyroid hormone receptor β-
472 dependent expression of a potassium conductance in inner hair cells at the onset of
473 hearing. *Proceedings of the National Academy of Sciences* **95**, 15758–15762 (1998).
474 9. S. S. Spicer, B. A. Schulte, The fine structure of spiral ligament cells relates to ion return
475 to the stria and varies with place-frequency. *Hear Res* **100**, 80–100 (1996).
476 10. Y. Cazals, *et al.*, KCNK5 channels mostly expressed in cochlear outer sulcus cells are
477 indispensable for hearing. *Nat Commun* **6**, 8780 (2015).
478 11. M. Galic, W. Giebel, An electron microscopic study of the function of the root cells in
479 the external spiral sulcus of the cochlea. *Acta Otolaryngol Suppl* **461**, 1–15 (1989).
480 12. D. J. Jagger, G. Nevill, A. Forge, The Membrane Properties of Cochlear Root Cells are
481 Consistent with Roles in Potassium Recirculation and Spatial Buffering. *J Assoc Res*
482 *Otolaryngol* **11**, 435–48 (2010).
483 13. D. J. Jagger, A. Forge, The enigmatic root cell - emerging roles contributing to fluid
484 homeostasis within the cochlear outer sulcus. *Hear Res* **303**, 1–11 (2013).
485 14. M. S. Deol, An Experimental Approach to the Understanding and Treatment of
486 Hereditary Syndromes with Congenital Deafness and Hypothyroidism. *Journal of*
487 *Medical Genetics* **10**, 235 (1973).
488 15. I. J. Karolyi, *et al.*, Dietary thyroid hormone replacement ameliorates hearing deficits in
489 hypothyroid mice. *Mamm Genome* **18**, 596–608 (2007).
490 16. A. Uziel, C. Legrand, A. Rabie, Corrective effects of thyroxine on cochlear abnormalities
491 induced by congenital hypothyroidism in the rat. I. Morphological study. *Brain research*
492 **351**, 111–22 (1985).
493 17. A. J. Duvall III, The ultrastructure of the external sulcus in the guinea pig cochlear duct.
494 *The Laryngoscope* **79**, 1–29 (1969).
495 18. D. J. Lim, M. Anniko, Developmental morphology of the mouse inner ear. A scanning
496 electron microscopic observation. *Acta Otolaryngol Suppl* **422**, 1–69 (1985).
497 19. R. Shodo, M. Hayatsu, D. Koga, A. Horii, T. Ushiki, Three-dimensional reconstruction of
498 root cells and interdental cells in the rat inner ear by serial section scanning electron

microscopy. *Biomed Res* **38**, 239–248 (2017).
20. Z. Xie, *et al.*, ZBTB20 is essential for cochlear maturation and hearing in mice. *Proceedings of the National Academy of Sciences* **120**, e2220867120 (2023).
21. L. Ng, *et al.*, Age-Related Hearing Loss and Degeneration of Cochlear Hair Cells in Mice Lacking Thyroid Hormone Receptor beta1. *Endocrinology* **156**, 3853–65 (2015).
22. S. Gu, *et al.*, Characterization of rare spindle and root cell transcriptional profiles in the stria vascularis of the adult mouse cochlea. *Sci Rep* **10**, 18100 (2020).
23. D. J. Bradley, H. C. Towle, W. S. Young, Alpha and beta thyroid hormone receptor (TR) gene expression during auditory neurogenesis: evidence for TR isoform-specific transcriptional regulation in vivo. *Proc Natl Acad Sci U S A* **91**, 439–443 (1994).
24. P. Jean, *et al.*, Single-cell transcriptomic profiling of the mouse cochlea: An atlas for targeted therapies. *Proceedings of the National Academy of Sciences* **120**, e2221744120 (2023).
25. O. Sawant, *et al.*, Light-Regulated Thyroid Hormone Signaling Is Required for Rod Photoreceptor Development in the Mouse Retina. *Invest Ophthalmol Vis Sci* **56**, 8248–8257 (2015).
26. A. Uziel, C. Legrand, A. Rabie, Corrective effects of thyroxine on cochlear abnormalities induced by congenital hypothyroidism in the rat. I. Morphological study. *Brain Res* **351**, 111–122 (1985).
27. B. J. Walters, J. Zuo, Postnatal development, maturation and aging in the mouse cochlea and their effects on hair cell regeneration. *Hear Res* **297**, 68–83 (2013).
28. W. Liu, C. Wang, H. Yu, S. Liu, J. Yang, Expression of acetylated tubulin in the postnatal developing mouse cochlea. *European journal of histochemistry : EJH* **62** (2018).
29. Y. Yan, *et al.*, Hepatic thyroid hormone signalling modulates glucose homeostasis through the regulation of GLP-1 production via bile acid-mediated FXR antagonism. *Nat Commun* **13**, 6408 (2022).
30. S. Liu, *et al.*, Triiodothyronine (T3) promotes brown fat hyperplasia via thyroid hormone receptor α mediated adipocyte progenitor cell proliferation. *Nat Commun* **13**, 3394 (2022).
31. J. M. Hebert, S. K. McConnell, Targeting of cre to the Foxg1 (BF-1) locus mediates loxP recombination in the telencephalon and other developing head structures. *Dev Biol* **222**, 296–306 (2000).
32. M. H. Song, *et al.*, Pou3f4 deficiency causes defects in otic fibrocytes and stria vascularis by different mechanisms. *Biochemical and Biophysical Research Communications* **404**, 528–533 (2011).
33. I. E. Royaux, *et al.*, Localization and functional studies of pendrin in the mouse inner ear provide insight about the etiology of deafness in pendred syndrome. *J Assoc Res Otolaryngol* **4**, 394–404 (2003).
34. R. S. Kimura, Sensory and accessory epithelia of the cochlea. *Friedmann, I And J Ballantyne (Ed) Ultrastructural Atlas Of The Inner Ear Ix++329p Butterworths*, London, England; Boston, Mass , Usa Illus P101-132 (1984).
35. A. Uziel, C. Legrand, M. Ohresser, M. Marot, Maturational and degenerative processes in the organ of Corti after neonatal hypothyroidism. *Hear Res* **11**, 203–218 (1983).
36. D. S. Sharlin, *et al.*, Deafness and loss of cochlear hair cells in the absence of thyroid

543 hormone transporters Slc16a2 (Mct8) and Slc16a10 (Mct10). *Sci Rep* **8**, 4403 (2018).
544 37. X. Hao, *et al.*, Sox10 expressing cells in the lateral wall of the aged mouse and human
545 cochlea. *PLoS One* **9**, e97389 (2014).
546 38. H. Hibino, F. Nin, C. Tsuzuki, Y. Kurachi, How is the highly positive endocochlear potential
547 formed? The specific architecture of the stria vascularis and the roles of the ion-
548 transport apparatus. *Pflugers Arch* **459**, 521–33 (2010).
549 39. H. J. Kim, *et al.*, Precise toxigenic ablation of intermediate cells abolishes the "battery"
550 of the cochlear duct. *J Neurosci* **33**, 14601–14606 (2013).
551 40. P. Wangemann, Supporting sensory transduction: cochlear fluid homeostasis and the
552 endocochlear potential. *J Physiol* **576**, 11–21 (2006).
553 41. M. Ando, S. Takeuchi, Immunological identification of an inward rectifier K+ channel
554 (Kir4.1) in the intermediate cell (melanocyte) of the cochlear stria vascularis of gerbils
555 and rats. *Cell Tissue Res* **298**, 179–183 (1999).
556 42. D. C. Marcus, T. Wu, P. Wangemann, P. Kofuji, KCNJ10 (Kir4.1) potassium channel
557 knockout abolishes endocochlear potential. *Am J Physiol Cell Physiol* **282**, C403-407
558 (2002).
559 43. T. P. Kerr, M. D. Ross, S. A. Ernst, Cellular localization of Na+,K+-ATPase in the
560 mammalian cochlear duct: significance for cochlear fluid balance. *Am J Otolaryngol* **3**,
561 332–338 (1982).
562 44. M. J. Dixon, *et al.*, Mutation of the Na-K-Cl Co-Transporter Gene Slc12a2 Results in
563 Deafness in Mice. *Human Molecular Genetics* **8**, 1579–1584 (1999).
564 45. M. C. Casimiro, *et al.*, Targeted disruption of the Kcnq1 gene produces a mouse model
565 of Jervell and Lange-Nielsen Syndrome. *Proc Natl Acad Sci U S A* **98**, 2526–2531 (2001).
566 46. M. Sakagami, *et al.*, Cellular localization of rat Isk protein in the stria vascularis by
567 immunohistochemical observation. *Hear Res* **56**, 168–172 (1991).
568 47. K. P. Steel, C. Barkway, Another role for melanocytes: their importance for normal stria
569 vascularis development in the mammalian inner ear. *Development* **107**, 453–463 (1989).
570 48. M. Cohen-Salmon, *et al.*, Targeted Ablation of Connexin26 in the Inner Ear Epithelial
571 Gap Junction Network Causes Hearing Impairment and Cell Death. *Current Biology* **12**,
572 1106–1111 (2002).
573 49. J. Sun, *et al.*, Cochlear gap junctions coassembled from Cx26 and 30 show faster
574 intercellular Ca2+ signaling than homomeric counterparts. *American Journal of*
575 *Physiology-Cell Physiology* **288**, C613–C623 (2005).
576 50. S. Ahmad, S. Chen, J. Sun, X. Lin, Connexins 26 and 30 are co-assembled to form gap
577 junctions in the cochlea of mice. *Biochemical and Biophysical Research Communications*
578 **307**, 362–368 (2003).
579 51. A. Uziel, Periods of sensitivity to thyroid hormone during the development of the organ
580 of Corti. *Acta Otolaryngol Suppl* **429**, 23–27 (1986).
581 52. B. Roth, V. Bruns, Postnatal development of the rat organ of Corti. I. General morphology,
582 basilar membrane, tectorial membrane and border cells. *Anat Embryol (Berl)* **185**, 559–
583 69 (1992).
584 53. D. J. Jagger, A. Forge, Connexins and gap junctions in the inner ear--it's not just about
585 K(+) recycling. *Cell Tissue Res* **360**, 633–44 (2015).
586 54. D. J. Jagger, A. Forge, Compartmentalized and signal-selective gap junctional coupling

in the hearing cochlea. *J Neurosci* **26**, 1260–8 (2006).
55. F. Nin, *et al.*, The endocochlear potential depends on two K+ diffusion potentials and an electrical barrier in the stria vascularis of the inner ear. *Proceedings of the National Academy of Sciences* **105**, 1751–1756 (2008).
56. G. Rickheit, *et al.*, Endocochlear potential depends on Cl- channels: mechanism underlying deafness in Bartter syndrome IV. *The EMBO journal* **27**, 2907–17 (2008).
57. P. Wangemann, J. Liu, D. C. Marcus, Ion transport mechanisms responsible for K+ secretion and the transepithelial voltage across marginal cells of stria vascularis in vitro. *Hearing Research* **84**, 19–29 (1995).
58. S. S. Spicer, B. A. Schulte, The fine structure of spiral ligament cells relates to ion return to the stria and varies with place-frequency. *Hear Res* **100**, 80–100 (1996).
59. N. Adachi, *et al.*, The mechanism underlying maintenance of the endocochlear potential by the K+ transport system in fibrocytes of the inner ear. *J Physiol* **591**, 4459–4472 (2013).
60. D. N. Furness, Forgotten Fibrocytes: A Neglected, Supporting Cell Type of the Cochlea With the Potential to be an Alternative Therapeutic Target in Hearing Loss. *Front Cell Neurosci* **13**, 532 (2019).
61. H. Davis, A model for transducer action in the cochlea. *Cold Spring Harb Symp Quant Biol* **30**, 181–190 (1965).
62. I. J. Russell, Origin of the receptor potential in inner hair cells of the mammalian cochlea--evidence for Davis' theory. *Nature* **301**, 334–336 (1983).
63. H. Liu, *et al.*, Organ of Corti and Stria Vascularis: Is there an Interdependence for Survival? *PLoS One* **11**, e0168953 (2016).
64. H. Liu, *et al.*, Molecular and cytological profiling of biological aging of mouse cochlear inner and outer hair cells. *Cell Rep* **39**, 110665 (2022).
65. P. Soriano, Generalized lacZ expression with the ROSA26 Cre reporter strain. *Nat Genet* **21**, 70–71 (1999).
66. J. M. Hebert, S. K. McConnell, Targeting of cre to the Foxg1 (BF-1) locus mediates loxP recombination in the telencephalon and other developing head structures. *Dev Biol* **222**, 296–306 (2000).
67. Z. Xie, *et al.*, Zinc finger protein ZBTB20 is a key repressor of alpha-fetoprotein gene transcription in liver. *Proc Natl Acad Sci U S A* **105**, 10859–10864 (2008).
68. Y. Li, H. Liu, X. Zhao, D. Z. He, Endolymphatic Potential Measured From Developing and Adult Mouse Inner Ear. *Front Cell Neurosci* **14**, 584928 (2020).
69. Z. Xie, *et al.*, Zbtb20 is essential for the specification of CA1 field identity in the developing hippocampus. *Proc Natl Acad Sci U S A* **107**, 6510–6515 (2010).

Figures and figure legends

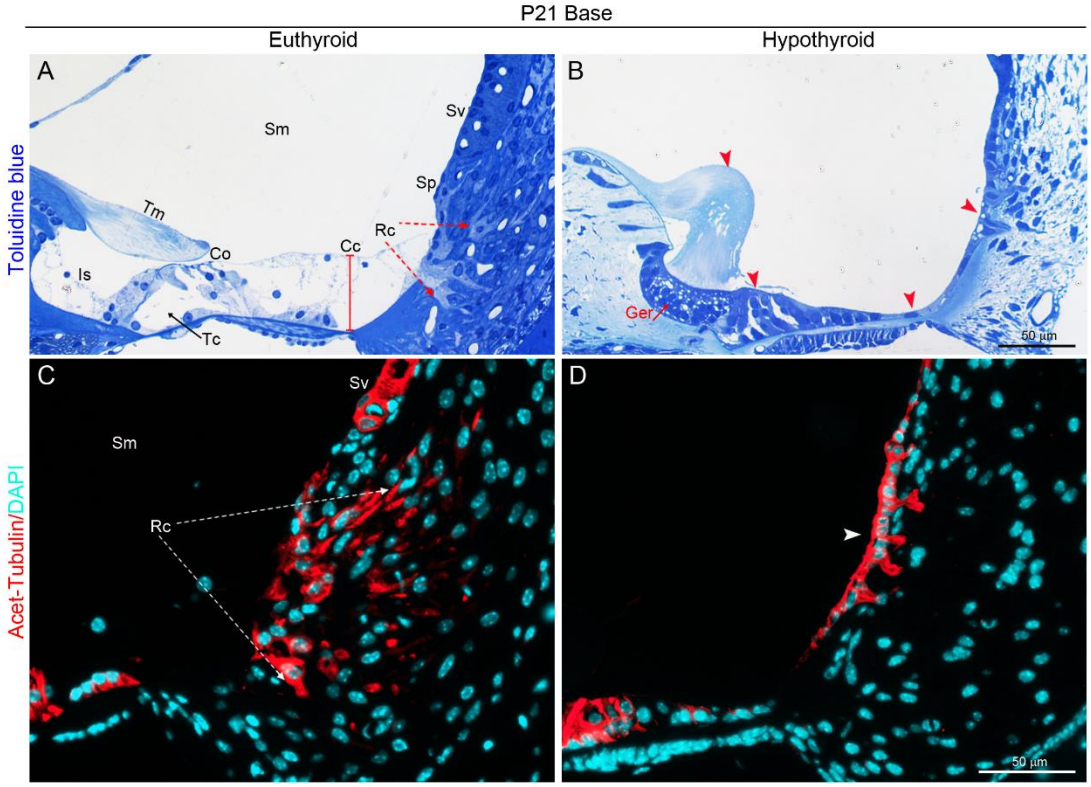

Fig. 1. Hypothyroidism causes the disruption in the outer sulcus development. (A and B) Representative Toluidine Blue-stained sections of cochlear upper basal turns from euthyroid (**A**) or methimazole (MMI)-induced hypothyroid mice (**B**) at P21. (**A**) Euthyroid cochlea showing a mature outer sulcus characterized by root cells (Rc. indicated by red arrows) with numerous bright branched processes extending deep into the spiral ligament and upward to behind the spiral prominence (Sp). The surfaces of root cell bodies were covered by enlarged Claudius cells (Cc, red bar indicating its height). Co, the organ of Corti; Is, inner sulcus; Sm, scala media; Sv, stria vascularis; Tc, tunnel of Corti, Tm, tectorial membrane. (**B**) The hypothyroid outer sulcus was consist of a simple layer of cells with a few short processes bordering the scala media, continued with the flattened Claudius cells. Red arrow heads indicate defects including a distorted Tm, an abnormal persistence of GER, closure of tunnel of Corti, and a flattened border region. (**C** and **D**) Immunostaing showing extensive acetyl-α-tubulin positive (red) root cell processes extending into the inferior region of euthyroid spiral ligament at P21 (**C**) but only a few short processes observed in the luminal surface of hypothyroid counterparts (indicated by white arrow head) (**D**). Bars = 50 µm for all panels. Nuclei were stained with DAPI (turquoise). n = 4 mice/ group. Scale bars in all panels = 50 µm. Abbreviations are consistent across figures in this manuscript.

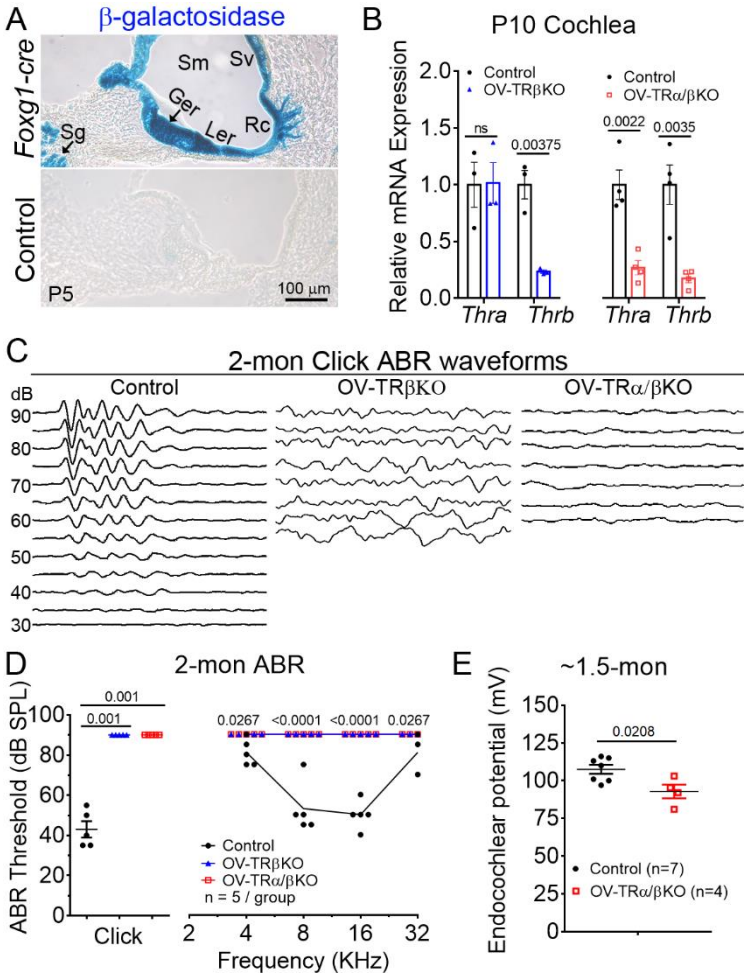

Fig.2 Cochlea-selective deletion of all known TRs causes deafness and a compromised EP value. (A) X-gal staining showing *Foxg1-Cre* recombinase activity was specifically confined in cochlear epithelium lining the scala media (Sm) at P5, including GER, LER (the lesser epithelial ridge), root cells (Rc), stria vascularis (Sv), as well as spiral ganglions (Sg). n = 3 mice/group. Bar = 100 μm. **(B)** QRT-PCR analysis showing relative mRNA expression levels of *Thra* and *Thrb* in control, OV-TRβKO, or OV-TRα/βKO cochlea at P10. n = 4 mice/group. **(C)** Representative ABR waveforms obtained to broadband click stimuli from control, OV-TRβKO and OV-TRα/βKO mice at 2 months (mon) of age. Sound pressure levels (SPL) were indicated in decibels (dB). (**D**) Averaged thresholds of Click- and Pure tone-evoked ABRs from control, OV-TRβKO and OV-TRα/βKO mice at 2 mon. n = 5 mice/group. (**E**) OV-TRα/βKO mice showing a reduced endocochlear potential (EP) in the basal cochlear turns compared with control mice at around 1.5 mon. The number of animals tested was indicated. All values are represented as mean ± SEM. A two-tailed Student's t-test was used for compare differences in relative mRNA expression levels, or EP values. A Kruskal-Wallis test (a nonparametric version of the ANOVA) with Dunn's multiple comparisons test and a two-way ANOVA followed by post-hoc pairwise tests (Bonferroni method) were used to compare differences in Click- and Pure tone-evoked ABR thresholds, respectively. *P* Values were indicated in each graphs. ns, not significant.

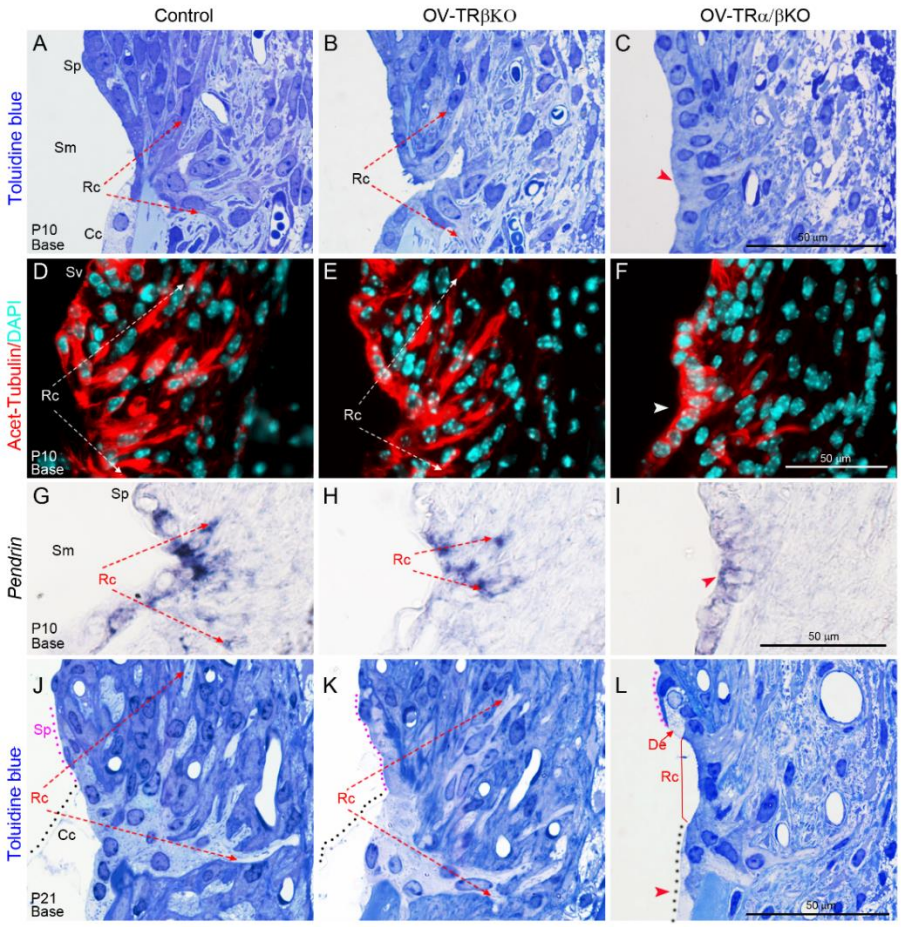

Fig. 3. Cochlea-selective deletion of all known TRs retards root cell development. **(A-C)** Representative Toluidine Blue-stained sections showing developing root cells (Rc) infiltrating deep into the spiral ligament(indicated by red arrows) from control **(A)** or OV-TRβKO **(B)** but mainly present in the luminal surface of scala media (Sm) in OV-TRα/βKO pups at P10 **(C)**. **(D-F)** Immunostaining showing extensive acetyl-α-tubulin positive (red) root cell processes within control **(D)** or OV-TRβKO **(E)** spiral ligament at P10, whereas only a few detected in OV-TRα/βKO counterparts **(F).** Nuclei were stained with DAPI (turquoise). n = 4 mice/ group. **(G-I)** In situ hybridization revealing that expression domain of *Pendrin* mRNA was extended into the inferior region of the spiral ligament in control **(G)** or OV-TRβKO **(H)** but were confined in the luminal surface in OV-TRα/βKO counterparts at P10 **(I)** . **(J-L)** Representative sections showing a mature outer sulcus in control **(J)** or OV-TRβKO **(K)** but not in OV-TRα/βKO **(L)** mice at P21. Both of control and OV-TRβKO mice showed elaborate root cell branches within the spiral ligament, the surfaces of which covered by the enlarged Claudius cells (Cc, outlined by black dots) and the spiral prominence epithelium (Sp, outlined by magenta dots) **(J and K),** whereas OV-TRα/βKO root cells (indicated by red bracket) were mainly bordering the scala media, separating a flattened Claudius cell (indicated by arrow head) and the spiral prominence epithelium. A cell with a pale-staining cytoplasm and nucleus (indicated by De) was detected in OV-TRα/βKO outer sulcus. The upper basal turns were shown for all panels and defects were indicated by arrow heads. Scale bars: 50 μm for all panels. n = 4 mice/group. See also *SI Appendix* Fig. S1and S2.

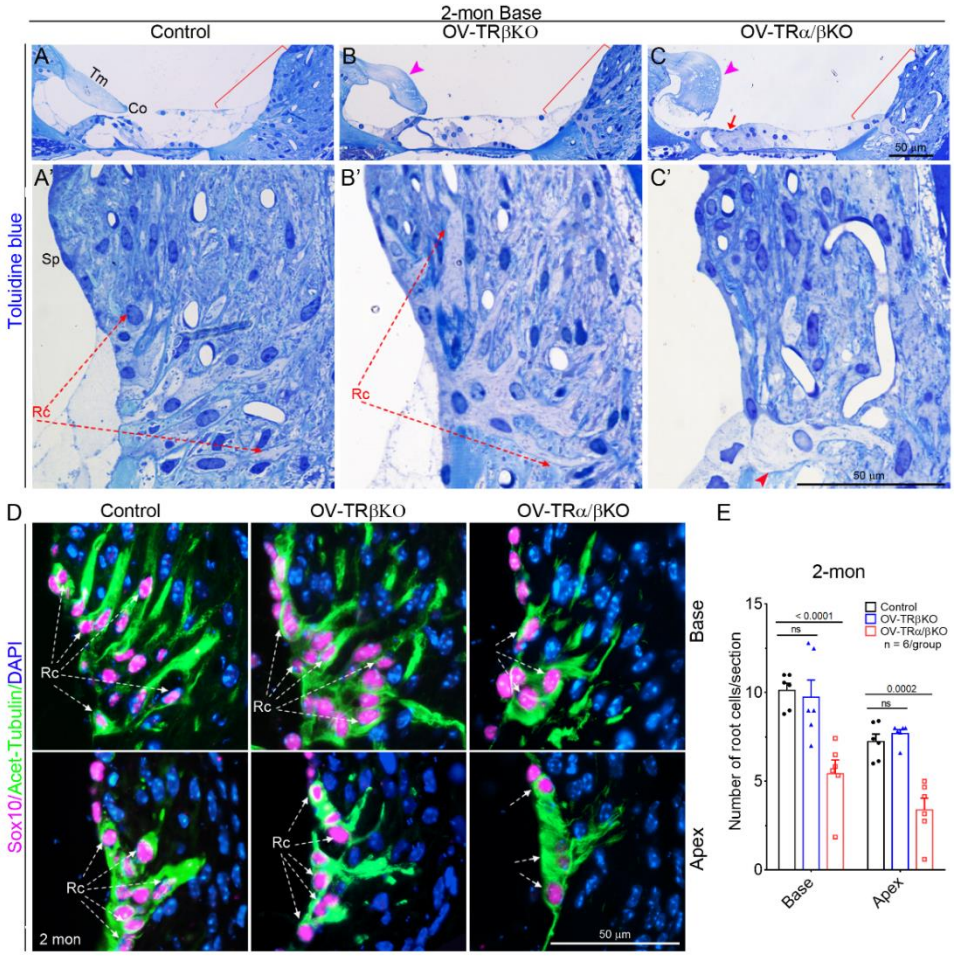

Fig. 4. Absence of all known TRs in cochlear epithelium causes early-onset root cell degeneration. (A-C) Representative Toluidine Blue-stained sections of upper basal turns from control **(A)**, OV-TRβKO **(B)**, and OV-TRα/βKO **(C)** cochleae at 2 months. Tectorial membrane (Tm, indicated by magenta arrow heads) malformations were observed in OV-TRβKO and OV-TRα/βKO, whereas a partially collapsed organ of Corti (Co) was only observed in OV-TRα/βKO mice, indicated by the red arrow in **(C)**. **(A')**, **(B')** and **(C')** are magnified views of outer sulcus regions indicated by red brackets in **(A)**, **(B)**, and **(C)**, respectively. Both of control and OV-TRβKO mice showed numerous branched root cells (Rc) within the spiral ligament, whereas OV-TRα/βKO counterparts showed reduced root cells with pale-staining processes and nuclei, indicated by red arrow head in (C'). Sp, Spiral prominence. n = 6 mice/group. **(D)** Double immunostaining showing Sox10 (magenta) and acetyl-α-tubulin (green) double-positive root cells within upper basal and apical turns from control, OV-TRβKO, or OV-TRα/βKO mice at 2 months. Nuclei were stained with DAPI (blue). n = 6 mice/group. (E) Bar graph showing reduced averaged number of Sox10 and acetyl-α-tubulin double-positive root cells in OV-TRα/βKO compared with that in control mice at 2 months. All values are represented as mean ±SEM. A two-way ANOVA followed by post-hoc pairwise tests (Bonferroni method) was used to compare differences. *P* Values were indicated in the graph. ns, not significant. Scale bars: 50 µm for all panels. See also *SI Appendix* Fig. S3.

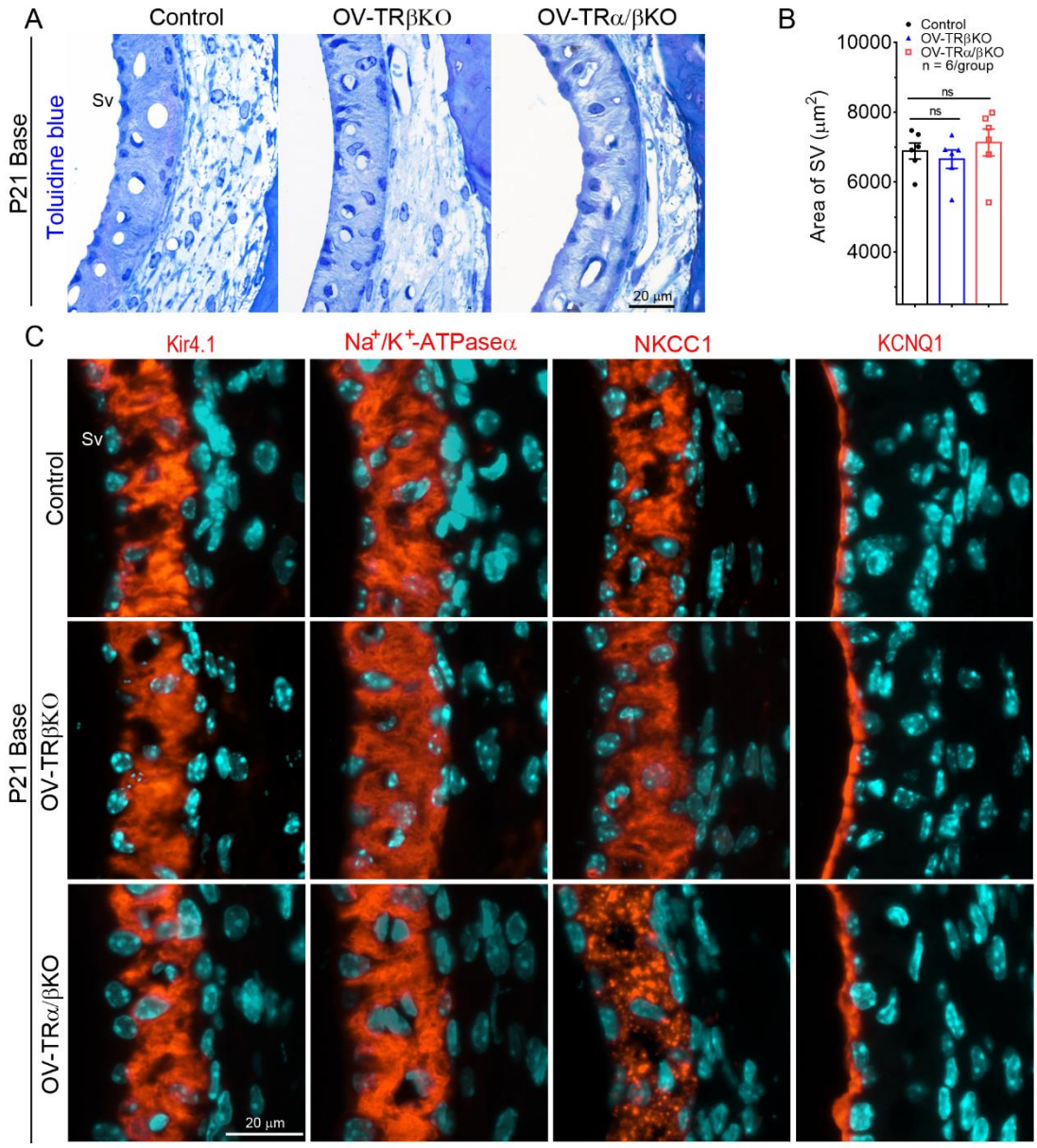

Fig. 5. Stria vascularis (SV) remains intact upon deletion of all known TRs in cochlea. (**A**) Representative Toluidine Blue-stained sections of cochlear upper basal turns showing comparable SV histology among Control, OV-TRβKO, and OV-TRα/βKO mice at P21. (**B**) Bar graph showing comparable SV areas among Control, OV-TRβKO, and OV-TRα/βKO mice at P21. n = 6 mice/group. Values are represented as mean ±SEM. A one-way ANOVA followed by post-hoc pairwise tests (Bonferroni method) was used to compare differences between the two groups. ns: not significant. (**C**) Immunostaining revealing comparable expressions of Kir4.1, Na+/K+-ATPase α, NKCC1, and KCNQ1 (red) among Control, OV-TRβKO, and OV-TRα/βKO mice at P21. n = 3 mice/group for each molecular marker. Scale bars: 20 μm for all panels. Nuclei were stained with DAPI (turquoise). See also *SI Appendix* Fig. S4.

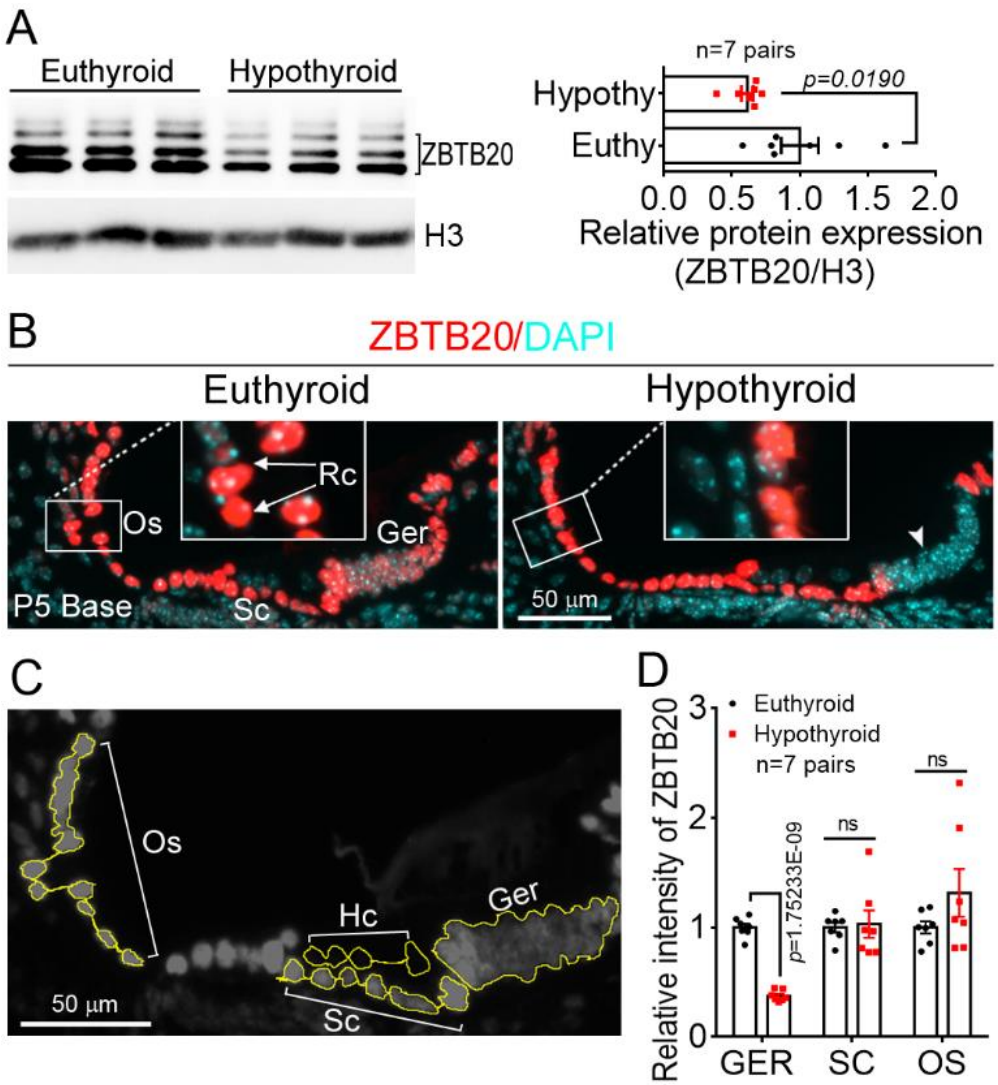

Fig. 6. TH signaling acts in parallel with ZBTB20 in the outer sulcus development. (A) Representative blot showing ZBTB20 protein was down regulated in cochleae from hypothyroid mice at P4 (left panel). Three isoforms of ZBTB20 can be recognized in each sample. Histone H3 is shown as a loading control. Quantitation of relative ZBTB20 expression is represented in the right panel, n = 7 mice/group. **(B)** Immunostaining showing ZBTB20 expression was down-regulated in GER (indicated by arrowhead) from hypothyroid cochlea, but was comparable in other cochlear epithelia including supporting cells (Sc) and outer sulcus (Os)/root cell (Rc), when compared to those from euthyroid counterparts at P5. Scale bar = 50 μm. **(C)** Representative image showing the quantification of ZBTB20 immunostaining intensity in indicated regions of the upper basal turn using Image J program (left panel). **(D)** Bar graph showing relative mean intensities of each sub-region normalized by those of hair cells (Hc, serve as the internal negative control) at the same turn. n = 7 mice/group. Values are expressed as mean ± SEM. Paired Student's t-test was used to compare the means of the two groups. ns, not significant. Uncropped western blot/gel images are shown in *SI Appendix* Fig. S5. See also *SI Appendix* Fig. S6 and S7.