Sox8 is sufficient to reprogram ectoderm into ear vesicles and associated neurons

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13 **The vertebrate inner ear arises from a pool of progenitors with the potential to give rise to all**
14 **the sense organs and cranial ganglia of the head¹⁻⁶. Here we explore the molecular**
15 **mechanisms that control ear specification from these precursors. Using a multi-omics**
16 **approach combined with loss-of-function experiments we identify a core transcriptional circuit**
17 **that imparts ear identity, along with non-coding elements that integrate this information. This**
18 **analysis places the transcription factor Sox8 at the top of the ear determination network.**
19 **Introducing Sox8 into cranial ectoderm not only converts non-ear cells into ear progenitors,**
20 **but also activates the cellular programmes for ear morphogenesis and neurogenesis. Thus,**
21 **Sox8 emerges as a master regulator of ear identity and may be a key factor for sense organ**
22 **cell reprogramming.**

Master regulators have the unique ability to reprogram cells from other cell types. They activate a set of transcription factors that in turn control the expression of proteins required for cell-specific functions. The classic example is the transcription factor MyoD, which can convert fibroblasts into myogenic cells⁷. From insects to vertebrates, the transcription factor Pax6 induces ectopic eyes when mis-expressed in non-eye cells⁸, however master regulators for other sense organs have not yet been discovered. Here we set out to identify such factors for the organ of hearing and balance, the vertebrate inner ear.

During development, the inner ear arises from the posterior border of the cranial neural plate. Here, precursors for neural, neural crest, ear and sensory ganglia are initially intermingled, but segregate over time^{9,10}. FGF signalling from adjacent tissues induces otic-epibranchial progenitors (OEPs), which subsequently converge to distinct territories: the otic and epibranchial placodes¹¹⁻¹⁵ (Fig. 1a, e). While epibranchial cells produce sensory ganglia, the otic placode invaginates to form a vesicle, which is then transformed into the inner ear, containing many specialised cell types and associated neurons. Using transcriptional profiling, we recently defined the first gene regulatory network for early ear development¹⁶. However, the key factors that impart ear character remain unknown as do the non-coding regions that integrate the process. Here we use single cell RNA-sequencing (scRNAseq) and pseudo-time analysis to predict transcriptional changes as progenitors undergo ear commitment. Using epigenomic profiling we also determine ear enhancers and their upstream regulators. To ensure transparency and reproducibility, we have developed a fully integrated workflow of the data analysis in Nextflow (available at <https://alexthiery.github.io/otic-reprogramming/>). Together with functional experiments, we identify a minimal transcriptional circuit that defines ear identity comprising Sox8, Pax2 and Lmx1a, with Sox8 at the top of the hierarchy. Introduction of Sox8 alone into cranial ectoderm is sufficient to trigger the ear programme, to initiate ear morphogenesis by forming ear vesicles and to activate neurogenesis. Thus, using a multi-omics approach we have uncovered Sox8 as a master regulator of ear fate, which may in the future allow reprogramming of specific sensory cell types.

Dynamic changes in gene expression characterise the transition from progenitor to otic commitment

To unravel the genetic hierarchy that controls how otic and epibranchial cells diverge, we first characterised the transcriptional profile of cells committed to each fate² (Fig1. a-d, Extended Data Fig. 1a, b). To label each cell population we used two enhancers driving EGFP, the novel otic Lmx1aE1 enhancer (see below) and the epibranchial enhancer Sox3U3¹⁷. Reporter constructs were electroporated into the OEP territory of somite stage (ss) 3-5 embryos. At ss18-21, EGFP⁺ cells were isolated by fluorescence-activated cell sorting (FACS) and processed for RNA-seq. Differential expression analysis identifies 103 and 319 genes upregulated in Lmx1aE1-EGFP and Sox3U3-EGFP expressing cells, respectively (log2FC > 1.5; adjusted p-value < 0.05; Fig. 1d; Extended Data Fig. 1c, d and Supplementary Table1). This allows us to define the transcriptional states of definitive otic and epibranchial cells.

Next, we investigated the transcriptional changes which take place as OEPs transition from a common progenitor population to definitive otic and epibranchial cells. *Pax2* is expressed throughout this time-window in both cell populations (Extended Data Fig. 1e, f)^{2,9}. Using a Pax2E1-EGFP reporter, we isolated single cells by FACS from consecutive stages of ear specification: OEP (ss8-9), early-placode (ss11-12) and late-placode stage (ss14-15) and processed them for single-cell-RNA-seq (scRNAseq; Fig. 1e, f). To characterise cellular diversity, we first looked at groups of genes co-expressed across the dataset (gene modules). These gene modules were identified in an unbiased manner through hierarchical clustering of gene-gene Spearman correlation coefficient¹⁸. Gene modules of interest were selected based on the presence of well-characterized makers for placodal, neural and neural-crest cells including the new otic or epibranchial genes identified above (Fig.1d). Initial cell clustering defines five major clusters (Extended Data Fig. 2a) to which we assigned identities using known markers (Fig. 1g-h, Extended Data Fig. 2c-f). Clusters C1 and C2 represent Pax2⁺/Six1⁺ cells expressing high levels of OEP and placodal makers (Fig. 1h, i; Extended Data Fig. 2c). Surprisingly, we also find two clusters with relatively few Pax2⁺ cells: one containing neural-like cells (C4; Sox21⁺) and another containing neural crest-like cells (C5; Pax7⁺) (Extended Data Fig. 2a, d, e). Since OEPs are mixed with neural and neural crest cells at early stages⁹, this observation

suggests that while cells in clusters C4/5 initially activate Pax2E1-EGFP they subsequently downregulate enhancer activity and *Pax2* expression.

To investigate the transcriptional dynamics accompanying otic and epibranchial fate decisions, we subset the placodal clusters (C1/2 in Fig. 1; Extended Data Fig. 2a). Re-clustering these cells using gene modules containing otic and epibranchial genes we obtained five placodal clusters (PC1-5; Fig. 1m, j, k; Supplementary Table 2). PC1 largely consists of only OEPs (ss8-9 cells), while the other clusters contain cells from both early and late placode stages (Fig. 1m, j, k). Indeed, PC1 is characterised by the expression of OEP genes (GM1-3; Fig. 1m), as well as sharing genes with the otic module GM5/6 and the epibranchial module GM7-9. In contrast, PC2/3 and PC4/5 are transcriptionally distinct from each other with profiles akin to otic (GM5/6) and epibranchial (GM7-9) cells, respectively. To explore the relationship between different cell clusters we organised cells along pseudo-time using Monocle2¹⁹. This analysis predicts that OEPs gradually split into one otic and one epibranchial branch, each composed of early and late placodal cells (Fig. 1n, o). Calculating RNA-velocity independently²⁰ and embedding the corresponding vector field onto the Monocle2 trajectory validates the directionality of predicted cell state transitions (Fig. 1p).

To explore dynamic changes of gene expression accompanying these inferred trajectories, we used Branch-Expression-Analysis-Modelling (BEAM)¹⁹. This identified groups of transcription factors expressed in OEPs prior to the branching point, which subsequently segregate into either the otic (e.g. *Sox8*, *Lmx1a*) or the epibranchial (e.g. *Foxi3*, *Tfap2e*) branch (Fig. 2a, Extended Data Fig. 3a). To quantify the changes in co-expression of otic and epibranchial genes, we assessed the proportion of co-expressing cells before and after the branching point. A two-tailed Wilcoxon rank sum test reveals significantly more cells co-expressing otic and epibranchial markers in OEPs than in epibranchial ($W=214$, $p=0.0013$) and otic cells ($W=235$, $p<0.0001$) after the branching point (Fig. 2b, c). Quantification of gene expression in the monocle trajectories and by *in situ* hybridization chain reaction (HCR)²¹ confirms that otic (*Sox8*, *Lmx1a*) and epibranchial (*Foxi3*, *Tfap2e*) transcripts overlap at ss8-9, and their expression resolves as both placodes are firmly established (Fig. 2d-i, Extended Data Fig. 3b-f).

Together, these results identify groups of transcription factors whose expression changes over time as otic and epibranchial precursors segregate. Some of these may play a key role in imparting ear identity.

Epigenomic profiling uncovers new regulatory elements and motifs in ear precursors

Transcription factors controlling cell fate choice regulate cell-specific downstream targets by interacting with tissue specific cis-regulatory enhancer elements. Active enhancers are regions of open chromatin flanked by nucleosomes enriched for histone 3 lysine-27 acetylation (H3K27ac), while actively transcribed genes are marked by H3K4me3²²⁻²⁵. We therefore profiled ss8-9 OEPs by ChIPseq for H3K27ac, H3K4me3 and the repressive mark H3K27me3 and determined chromatin accessibility by ATACseq. Overlapping H3K27ac and ATACseq data identifies 10969 regions with depleted H3K27me3 marks; average profiles show bimodal H3K27ac read distribution surrounding ATACseq peaks (Extended Data Fig. 4a). Of these just over 70% are intergenic or intronic representing putative enhancers (8316), while the remaining are close to transcription start sites (TSS; Extended Data Fig. 3b). We associated each putative enhancer to the nearest TSS of protein coding genes; GO term analysis of the corresponding genes returns MAP-kinase, Wnt and Notch signalling known to mediate ear induction, development and neurogenesis (Extended Data Fig. 4c)^{13,15}. We selected putative enhancers in the vicinity of ear-enriched genes to assess their activity *in vivo*, generated EGFP reporters and co-electroporated them with ubiquitously expressed mCherry into head-fold-stage chick embryos. RT-PCR-based assays²⁶ (not shown) and fluorescence microscopy confirm enhancer activity in ear progenitors and otic placodes (Fig. 3a, b; Extended Data Fig. 5-7). Next, we performed motif enrichment analysis of all ear enhancers to identify upstream regulators that may act as otic determinants. This reveals an over-representation of binding sites for Sox, TEAD and Six family members and for Tfp2a (Fig. 3c; Extended Data Fig. 4d). Of these, Tfp2a and Six proteins have previously been implicated in cranial placode development²⁷⁻³¹, confirming that this strategy can identify relevant regulatory factors.

We also exploited the idea that cell identity genes may be regulated by super-enhancers characterised by broad H3K27ac, while their gene bodies harbour broad H3K4me3³²⁻³⁴. Focusing on

OEP factors segregating to the ear lineage (Fig. 2a) that are also prominent in the otic GRN¹⁶, we find that the *Sox8* locus is marked with H3K4me3, while enhancers close to *Lmx1a*, *Zbtb16* and *Sox13* show broad H3K27ac marks (Fig. 3a, Extended data 4e, 6a, 7a). Together, this analysis points to *Lmx1a*, *Zbtb16* and members of the Sox transcription factor family as potential key regulators of ear identity.

Sox8, Lmx1a and Pax2 are core components of the ear determination network

Our transcriptome analysis identifies several Sox genes enriched in otic progenitors. Of these, *Sox3* and *Sox13* are expressed prior to ear specification, while *Sox9* and *-10* are activated later¹⁶. These genes are therefore unlikely to initiate the otic programme. In contrast, the SoxE group factor *Sox8* is highly enriched in OEPs prior to segregating to otic cells (Fig. 3d). *In situ* hybridisation reveals that *Sox8* begins to be expressed at 3ss, followed shortly thereafter by *Pax2*, *Zbtb16*, and *Lmx1a*, while *Foxg1*, *Soho1* and *Sox10* are activated later (Fig. 3d, Extended Data Fig. 5a).

To explore the regulatory interactions between the earliest OEP transcription factors, we systematically knocked down each one in turn and assayed the expression of all others as well as that of *Foxg1* and *Soho1* as a readout for otic identity using *in situ* hybridisation and RT-qPCR (Fig. 2e; Extended Data Fig. 5b, c). Control or antisense oligonucleotides targeting *Sox8*, *Pax2*, *Zbtb16* or *Lmx1a* were electroporated into future OEPs of head-fold-stage chick embryos and gene expression analysed at OEP stage (ss8-9). We find that *Sox8* is necessary for the expression of all assayed ear transcription factors. *Pax2* is required for the expression of *Zbtb16* and *Lmx1a*, which in turn is necessary for *Pax2*, suggesting that they act in a positive feedback loop. All gene expression changes can be rescued by co-electroporation of the appropriate full-length constructs (Extended Data Fig. 9). These experiments put *Sox8* at the top of the ear determination network forming a regulatory circuit with *Pax2* and *Lmx1a*.

Sox8 induces ectopic otic vesicles and vesicle-derived neurons

If *Sox8*, *Pax2* and *Lmx1a* indeed form a minimal circuit driving otic specification, they should be able to convert non-ear cells into cells with ear identity. We tested this hypothesis by electroporating a

combination of Sox8/Pax2/Lmx1a-mCherry-tagged constructs into head-fold-stage ectoderm not destined to contribute to the ear. After 72hrs we observe many otic vesicles scattered across the head ectoderm. Some of these contain neurofilament-positive neurons, with processes projecting out from the ectopic vesicle (Fig. 4a).

We next asked whether Sox8 alone can initiate the otic programme using the Lmx1aE1-EGFP enhancer and otic markers as a readout. We find that misexpression of Sox8-mCherry not only activates Lmx1aE1-EGFP in ectopic vesicles, but also the expression of *Pax2* and *Lmx1a*, and the ear marker *Soho-1* (Fig. 4b-d; Extended Data Fig. 10a). To assess to what extent Sox8 can confer ear identity, we isolated double-positive Sox8-mCherry/Lmx1a-EGFP cells from ss11-12 by FACS and compared their transcriptome with control ectoderm labelled with constitutive mCherry/EGFP. Differential expression analysis shows upregulation of 399 transcripts in comparison to controls, while 112 genes are downregulated ($\log_2FC > 1.5$; adjusted p-value < 0.05) (Fig. 4e, Extended Data Fig. 9b-d, Supplementary Table 3). Seventeen of the twenty seven upregulated transcription factors are known to be expressed in the otic placode or vesicle, while for the remaining ten no expression data are available (Supplementary Table 3). In contrast, among the downregulated genes are typical neural crest and forebrain transcription factors. The first cell types specified during ear formation are neurons of the cochlear-vestibular ganglion, we therefore asked whether Sox8 can also trigger neurogenesis. We find that Sox8-induced ectopic vesicles are associated with neurofilament-positive neurites generated from Sox8 expressing cells themselves (Fig. 4f, g). Together, these results show that Sox8 can activate the transcriptional program for ear identity in non-ear cells by direct lineage reprogramming *in vivo*.

Conclusion

We have established an integrated workflow for multi-omics data analysis to ensure accessibility and reproducibility. Using transcriptional profiling at single cell level, enhancer discovery and analysis, together with functional experiments, we have identified the critical components of the ear determination network: Sox8, Pax2 and Lmx1a (Fig. 4h). Sox8 lies at the top of the transcriptional hierarchy, and alone is sufficient to impart ear identity to cells otherwise destined to form head

epidermis, other sense organs or cranial ganglia. Previous findings have implicated Pax2 in otic placode development and patterning³⁵⁻³⁸ and Sox8 in regulating ear-specific enhancers³⁹⁻⁴¹. Sox proteins cooperate with a variety of transcription factors to exert their cell type specific function⁴². It is therefore tempting to speculate that, in analogy to the eye, where Sox2 cooperates with Pax6 to regulate lens-specific transcription⁴³, in the ear Sox8 might partner with Pax2.

Our results also show that OEPs initially express competing transcriptional programmes that resolve over time as otic and epibranchial cell states are established. We capture previously unknown gene modules that accompany this process as well as regulatory regions associated with otic-epibranchial specification. In turn, this information is critical to unravel the underlying gene regulatory networks and identify the transcription factor codes that determine cell identity in the cranial sensory nervous system. In the long term, this will enhance our ability to engineer specific sensory cell types for basic research and regenerative purposes.

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308 **Figures and Figure Legends**

322 **Figure 1. Transcriptomic characterisation of ear development.** a-c, *In ovo* electroporation (a) was
323 used to label and collect otic (b; *Lmx1aE1*-EGFP⁺) and epibranchial (c; *Sox3U3*-EGFP⁺) cells for bulk
324 RNAseq. d, Volcano plot showing genes differentially expressed (absolute log₂ fold-change >1.5 and
325 adjusted p-value < 0.05) in otic (orange) and epibranchial cells (green). e, f, Cells were collected for
326 scRNAseq after *ex ovo* electroporation of the Pax2E1-EGFP enhancer, which is active in OEPs, otic
327 and epibranchial placodes (f); cells were collected at the stages indicated. g-i, tSNE representation
328 of unsupervised hierarchical clustering of all cells (g-i) and of the placodal cell subset (j-l); cells were
329 colour-coded according to stage collected (g, j; OEP grey, ss11-12 blue, ss14-15 black), clusters (h,
330 k) and placodal markers *Six1* (i) and *Pax2* (l) expression. m, Heatmap showing partitioning of the
331 placodal subset (C1, C2 in h) into five clusters (PC1-5) based on gene modules (GM). Expression
332 profiles reveal that PC1 largely contains OEP-like cells, while PC2/ and PC4/5 are composed of otic-
333 like and epibranchial-like cells. n-o, Pseudo-time ordering using Monocle2 shows trajectories
334 between stages (n) and clusters (o). Note: otic- and epibranchial-like cells segregate into two
335 branches. p, RNAvelocity vector field verifies the directional trajectories predicted by Monocle2.

Figure 2. Dynamic gene expression during OEP segregation into otic and epibranchial lineages. **a**, BEAM identifies genes regulated in a branch-dependant manner. **b**, Histogram showing the proportion of cells co-expressing genes that are expressed before the branching point, but segregate to the otic or epibranchial branch. Significantly more cells co-express such genes before the branching point than thereafter. Error bars indicate +/- 1 standard deviation. ** p-value < 0.01; *** p-value < 0.001. **c**, Dot plot for OEP, otic and epibranchial markers based on scRNAseq data. Expression level is indicated by colour intensity and gene expression frequency by dot size. **d**, **e**, A proportion of cells co-expresses otic (*Sox8*, *Lmx1a*) and epibranchial (*Foxi3*, *Ap2e*) markers prior to the branching point. **f-i**, *In situ* HCR (**f**, **h**) and intensity measurements (**g**, **i**) along the medial (M) to lateral (L) axis of the OEP-domain confirms gradual segregation of OEP gene expression as distinct otic and epibranchial cell populations emerge.

Figure 3. Chromatin accessibility profile of OEPs identifies putative regulatory regions. a, Genome browser view of ATAC, H3K27ac and H3K27me3 profiles in OEPs at the Lmx1a locus. Cloned putative enhancer is shown in green. **b,** Coelectroporation of Lmx1aE1-EGFP reporter (green) and constitutive mCherry (magenta) reveals *in vivo* enhancer activity in the otic placode. **c,** Motif enrichment analysis of all identified enhancers. **d,** Diagram showing the activation of expression of potential regulators of otic identity during specification and commitment stages. **e,** Knock-down of selected transcription factors using fluorescein labelled antisense oligonucleotides (aON; green) lead to downregulation of otic markers as identified by *in situ* hybridisation (blue).

Figure 4. Direct reprogramming of ectoderm by Sox8. **a**, Co-electroporation of mCherry-tagged vectors containing the full-length sequence of Sox8/Pax2/Lmx1a (magenta) generate ectopic vesicles across the cranial ectoderm after 72hrs culture. Neurofilament antibody staining (NF; green) shows axonal projections from the vesicle. **b-d**, Overexpression of Sox8 (Sox8 OE) alone activates Lmx1aE1-EGFP (green) and otic marker expression on the electroporated, but not on the control side (Ctrl). **e**, RNAseq of Sox8 and control electroporated cranial ectoderm; volcano plot shows enrichment of otic genes in the Sox8 OE (absolute log2 fold-change >1.5 and adjusted p-value < 0.05). **f**, Sox8-mCherry overexpression (magenta) alone generates ectopic otic vesicles with neuronal projections (green), while controls do not (**g**). **h**, BioTapestry model showing the minimal transcriptional circuit for otic specification with Sox8 at the top of the hierarchy.

Methods

Expression and enhancer constructs

Putative enhancers were amplified from chick genomic DNA and cloned into pTK-EGFP reporter vectors after digestion with XcmI²⁶. To generate expression constructs total RNA was isolated from HH8-12 chick embryos with RNAqueous-Micro Total RNA Isolation Kit (Thermo Fisher Scientific), reverse transcribed using Superscript II reverse transcriptase (Thermo Fisher Scientific, 180644-014) and oligo-dT primer. Specific primers were used to amplify the full-length coding sequence of Sox8, Pax2, Lmx1a and Zbtb16 and PCR products were cloned into pCAB-IRES-mCherry. All sequences were verified by Sanger sequencing.

Chick embryos, electroporation and culture

Fertilised hens' eggs (Stewart, Norfolk UK) were incubated at 38°C and staged according to Hamburger and Hamilton (HH)⁴⁴. All experiments were performed on embryos younger than 12 days, thus were not regulated by the Animals Scientific Procedures Act 1986.

OEPs for bulk RNAseq were labelled using *in ovo* electroporation⁴⁵; eggs were incubated until the 3-6 somite stage (ss), pTK-Lmx1aE1-EGFP or pTK-Sox3U3-EGFP plasmids (1µg/µl) were injected targeting the OEP territory. Electroporation using Ovodyne electroporator (TSS20, Intracel) was performed with five 50ms pulses of 8V at 100ms intervals. After incubation until ss18-21, embryos were collected in PBS and processed for bulk RNAseq.

For *ex ovo* culture, embryos were harvested using filter paper rings⁴⁶ and cultured on egg albumen; for long-term culture (72hrs) the 'modified Cornish pasty' method⁴⁷ was used. *Ex ovo* electroporation was performed to collect cells for scRNAseq, for knock-down, rescue and overexpression experiments. The posterior placodal region or the cranial surface ectoderm of HH6 embryos was targeted for electroporation by injecting plasmid DNA at 1µg/ul (pTK-Pax2E1-EGFP to label OEPs; pCES-Sox8-mCherry + pTK-Lmx1aE1-EGFP, pCES-Sox8-mCherry + pCES-Lmx1a-mCherry + pCES-Pax2-mCherry; pCES-mCherry + pCES-EGFP for misexpression), control or antisense-oligonucleotides (aON; GeneTools; 0.75mM with 0.3µg/µl carrier DNA), or a combination of aON and expression construct (0.75mM aON + 0.75µg/µl plasmid). Oligonucleotides used were Sox8: 5'-

CTCCTCGGTCATGTTGAGCATTGG-3'³⁹, Pax2: 5'-GGTCTGCCTTGCAGTGCATATCCAT-3'³⁷,
Lmx1a: 5'-CCTCCATCTTCAAGCCGTCCAGCAT-3'¹⁶, Zbtb16: 5'-
GTCAAATCCATAGCACTCCCGAGGT-3', Sox13: 5'-CTCCTCATGGACATCCATTTTCATTC-3' and
control 5'-ATGGCCTCGGAGCTGGAGAGCCTCA-3'. Electroporation was performed in a chamber
using five 5V pulses of 50ms in 100ms intervals⁴⁵.

Whole mount and hybridization chain reaction fluorescent *in situ* hybridisation

In situ hybridization was carried out following previously described protocols⁴⁸. Whole-mount pictures
were taken using an Olympus SZX12 with a Retiga2000R camera and Q-Capture Pro7 software.

HCR v3 was performed using the Molecular Technologies protocol²¹. Briefly, embryos were
fixed in 4% PFA for one hour at room temperature, dehydrated in a series of methanol in PBT and
stored overnight at -20°C. After rehydration and proteinase-K treatment (20mg/ml; 3 min) embryos
were post-fixed in 4% PFA for 20 min. Embryos were then washed on ice in PBS, 1:1 PBT/5X SSC
(5X sodium chloride sodium citrate, 0.1% Tween-20) and 5X SSC for 5 min each. Pre-hybridization
in hybridisation buffer was performed for 5 min on ice, followed by 30 minutes at 37°C. Embryos were
hybridised overnight at 37°C with probes at 4pmol/ml in hybridization buffer. After four 15 min washes
with probe wash buffer at 37°C, preamplification was carried out in amplification buffer for 5 min at
room temperature. Hairpins were prepared individually at 30pmol final concentration; they were
incubated at 95°C for 90 seconds followed by cooling to room temperature for 30 min, protected from
light. Cooled hairpins were added to 500µl amplification buffer, embryos were incubated in hairpins
overnight at room temperature followed by two 5 min and two 30 min washes in 5X SSC. After a 5
min incubation in DAPI (10 mg/ml), they were washed three times for 10 min with 5X SSC, before
being imaged using Leica SP5 laser scanning confocal inverted microscope using the LAS AF
software.

For HCR image analysis, Z-stacks were collected for 50-70µm; figures show projections of all
stacks. Images were processed using ImageJ and the ImageJ Plot Profile tool was used to calculate
intensity plots. In brief, an optical section in the centre of the placode territory was selected using the

Pax2 channel as a reference. The Pax2 channel was added to the Sox8, Lmx1a, Foxi3 or Tfp2e channels, respectively. Intensity values were then calculated across the area of interest and plotted.

Whole mount immunostaining

Embryos were collected in PBS, fixed for 25 minutes at room temperature in 4% PFA, washed in PBS-Tx (PBS + 0.2% Triton X-100) and blocked in 5% goat serum in PBS-Tx for 3–5 h at room temperature. Embryos were then incubated in primary antibody diluted in blocking buffer for 24–72 hours at 4°C. Primary antibodies were rabbit anti-mCherry (1:200; Abcam ab167453), mouse anti-NF (1:100; Thermo Fisher Scientific 13-0700), rabbit anti-GFP (1:500; Abcam a11122) or mouse anti-GFP (1:1000; Molecular Probes A11120). After five 60 min washes and one overnight wash in PBS-Tx, embryos were incubated in secondary antibodies (1:800) at 4°C overnight. Secondary antibodies used were goat anti-rabbit IgG Alexa Fluor 488 (Thermo Fisher Scientific, A11036), donkey anti-rabbit Alexa Fluor 568 (Thermo Fisher Scientific, A11001), goat anti mouse IgG Alexa Fluor 488 (Molecular Probes A11001) and goat anti mouse IgG Alexa Fluor 568 (Molecular Probes A11004). Embryos were then were briefly incubated in PBS containing 10 mg/ml DAPI and washed at least five times in PBS-Tx before being mounted on slides and imaged using Leica SP5 laser scanning confocal inverted microscope using a 10x objective or an Olympus SZX12 with a Retiga2000R camera and Q-Capture Pro7 software. Confocal whole mount images in the manuscript are maximum intensity projections of embryo z-stacks. Sections were imaged using a 63x oil immersion objective and maximum intensity projections are shown.

Cryosectioning

Embryos were embedded in gelatine as previously described⁴⁹ and cryo-sectioned at 15–20µm using a Bright OTF5000 cryostat. Sections were mounted using Mowiol 4-88 (Sigma Aldrich, 81381) and imaged using Leica SP5 laser scanning confocal inverted microscope (LAS AF software) or a Zeiss Axiovert 200M microscope with a Hamamatsu C4742-95 camera and using OCULAR software.

Quantitative PCR (qPCR)

RNA from dissected otic tissue was isolated using the RNAqueous-Micro Kit (Ambion, AM1931) and reverse transcribed. Primers for target genes were designed with PrimerQuest (IDT). qPCR was performed using Aria Mx Real-Time System (Agilent Technologies) with SYBR green master mix (Roche, 64913850001). RT-qPCR for antisense oligonucleotide experiments was carried out with a minimum of three biological replicates; two sample Welch's t-tests with Benjamini-Hochberg multiple test correction were used to determine statistical significance between control and antisense oligonucleotide ΔC_t values. Relative expression ($2^{-\Delta\Delta C_t}$)⁵⁰ was calculated using the NormqPCR package in R. The geometric mean of Gapdh and Rplp1 expression, or Rplp1 expression alone, was used to normalise gene expression.

Bulk RNA sequencing

To label otic and epibranchial cells embryos were electroporated with Lmx1aE1-EGFP and Sox3U3-EGFP plasmids, respectively, and whole heads were used for cell collection. For overexpression experiments embryos were electroporated with Sox8-mCherry+Lmx1aE1-EGFP or with pCAB-mCherry+pCAB-EGFP, the endogenous otic placode and the trunk were removed before cell dissociation. Cells were dissociated in FACSmax cell dissociation solution (Ambion, T200100) containing papain (30U/mg, Sigma-Aldrich, 10108014001) for 20min at 37°C before being transferred to Hanks Balanced Solution without calcium and magnesium (HBSS, Life Technologies, 14185045) containing 5% heat-inactivated foetal-bovine-serum (FBS), rock inhibitor (10 μ M, Stemcell Technologies, Y-27632) and non-essential amino acids (Thermo Fisher Scientific, 11140035). Cells were disaggregated by pipetting, sequentially filtered through 0.35 μ m and 0.20 μ m filters (Miltenyi Biotech, 130-101-812). Pelleted cells were resuspended in 500 μ l HBSS and isolated by fluorescent activated cell sorting (FACS) using a BD FACS-Aria Diva. 2000 cells per biological replicate were collected, centrifuged at 200xg for 5 mins at 4°C, washed with PBS, and resuspended in lysis buffer. RNA was extracted using Ambion RNAqueous Micro Total RNA isolation kit (AM1931, ThermoFisher Scientific). RNA integrity was checked using Bioanalyser with Agilent RNA 6000 pico kit (Agilent Technologies, 5067-1513); samples with RIN >7 were processed for library preparation. Sequencing libraries were prepared using Nextera Sample low input kit (Illumina, 15028212) and sequenced using

75bp paired end reads on the Illumina HiSeq4000 platform. A minimum of three biological replicates were used for analysis.

Single-cell RNA extraction, library preparation and sequencing

HH6-7 embryos were electroporated with Pax2E1-EGFP to label OEPs; cells were dissociated as described above and 288 EGFP⁺ cells from each ss8-9, ss11-12 and ss14-15 were collected by FACS in 96-well plates. Sequencing libraries were prepared following the SmartSeq2 protocol⁵¹. Libraries were sequenced on the Illumina NextSeq500 platform using single end 75bp sequencing (ss8-9) or on the HiSeq4000 platform using paired end 75bp sequencing (ss11-12, ss14-15).

Nuclei isolation, ATAC library preparation and sequencing

The ATACseq library was prepared following published protocols⁵². Approximately 30 pieces of the OEP territory were dissected from ss8-9 embryos, dissociated with Dounce homogenizer (tight pestle) in lysis buffer (10mM Tris-HCl, pH7.4, 10mM NaCl, 3mM MgCl₂, 0.1% IGEPAL CA-630). Nuclei were pelleted at 4000 rpm at 4°C for 10 min. After removing the lysis buffer, 1.25μl Tn5 transposase (Illumina, FC-131-1024) were added to 25μl reaction volume and incubated at 37°C for 10 mins. Tagmented DNA was then purified with Mini Elute PCR purification kit (Qiagen, 28004) followed by 9 cycles of PCR enrichment using NEB High fidelity PCR kit (NEB, M0541S). The quality of ATAC libraries was assessed with Agilent Bioanalyzer with DNA High Sensitivity kit (Agilent Technologies, 5067-4627) and quantified with Kapa NGS library quantification kit (Kapa Biosystems, KK4824). The libraries were sequenced with Illumina HiSeq 2000/2500 in 2x100 cycles.

H3K27ac, H3K27me3 and H3K4me4 ChIP library preparation and sequencing

Approximately 200 pieces of OEP ectoderm were dissected from ss8-9 embryos. The tissue was dissociated in nuclei expulsion buffer (0.5% NP-40, 0.25% Triton X-100, 10 mM Tris-HCl, pH 7.5, 3mM CaCl₂, 0.25M sucrose, 1x protease inhibitor (Roche), 1mM DTT, and 0.2mM PMSF) using a Dounce homogenizer (loose pestle); crosslinking was performed in 1% formaldehyde for 9 minutes, followed by quenching with 1M glycine for 5 minutes. Cells were then pelleted and washed 3 times

with PBS containing protease inhibitor (Roche, 11873580001). The pellets were snap-frozen and stored at -80°C for later use. Crosslinked chromatin was fragmented by sonication in an ice bath (Misonix Q700 at 7 Amplitude, 5x40 seconds, 30 seconds on, 60 seconds off) and immunoprecipitated following the Nano-ChIP protocol⁵³. For chromatin immunoprecipitation (ChIP), the following antibodies were used anti-H3K27Ac (Abcam, ab4729), anti-H3K4me3 (Diagenode, A5051-001P) and anti-H3K27me3 (Millipore, 07449). Adaptors and primers from NEBNext library preparation kit (Illumina, E6040S) were used to prepare the library following NanoChip protocol⁵³. The library was enriched using 14 PCR cycles and quantified with Kapa NGS library quantification kit (Kapa Biosystems, KK4824) before pooling at a concentration of 20nM. Library quality was assessed with Agilent Bioanalyzer with DNA High Sensitivity kit (Agilent Technologies, 5067-4627) and quantified with Kapa NGS library quantification kit (Kapa Biosystems, KK4824). The libraries were sequenced with Illumina HiSeq 2000/2500 in 2x100 cycles.

High-Throughput-Sequencing-Data (HTSD) Analysis

All data alignment and downstream analysis was carried out using NF-core and custom Nextflow pipelines to allow full reproducibility. All code used, including Nextflow pipelines and downstream analysis, can be found at <https://github.com/alexthiery/otic-reprogramming>. Full detailed documentation for the pipeline is also available at <https://alexthiery.github.io/otic-reprogramming/>. A custom Docker container used for the downstream analysis pipeline can be found at https://hub.docker.com/repository/docker/alexthiery/otic-reprogramming-r_analysis. This also allows for interactive exploration of the data.

Bulk-RNA-seq

Bulk-RNA-seq data were processed and aligned to GalGal6 using the default NF-core RNA-seq (v2.0) pipeline⁵⁴ which uses the STAR aligner. Downstream differential expression analysis (Lmx1aE1-EGFP vs Sox3U3-EGFP; Sox8OE vs ControlOE) was carried out with the DESeq2 package in R⁵⁵. Adjusted p-values were calculated using the default DESeq2 multiple test correction (Benjamini-

Hochberg). Differentially expressed transcripts were determined by an absolute log2 fold-change >1.5 and adjusted p-value < 0.05.

Single-cell-RNA-seq alignment

SmartSeq2 single-cell-RNA-seq reads were aligned and processed using a custom Nextflow DSL2 pipeline. Adaptor sequences were trimmed using Cutadapt (v2.10). HISAT2 (v2.2.1) was then used to build a genome index from GalGal6 (amended to include a GFP sequence), before extracting splice sites from the GTF and aligning reads. Read counts were obtained using HTSeq (v0.12.4). BAM files from HISAT2 were also passed to Velocyto (v0.17)²⁰ in order to get spliced and unspliced expression matrices for further downstream analysis.

Single-cell-RNA-seq data analysis

Downstream data analysis was carried out primarily using the Antler R package (version: Development2019)¹⁸. We excluded from the dataset: cells which expressed fewer than 1k genes or fewer than 500k reads, cells with more than 6% reads from mitochondrial genes, genes which were expressed in fewer than 3 cells and genes with CPM < 10.

Identification of gene modules

To identify clusters of genes with correlated expression unbiasedly (gene modules), we used the Antler *identifyGeneModules* function. Genes which had a Spearman correlation greater than 0.3 with at least 3 other genes were first removed. Genes were then hierarchically clustered iteratively into gene modules and filtered. Gene modules were filtered based on the minimum expression level (5 CPM) and the proportion (0.4) of cells expressing a gene module. The number of final gene modules was set to 40 to achieve reasonably large clusters, which broadly characterise cell type diversity across the dataset. Gene modules were then filtered based on the presence of genes with known expression profiles in ectodermal derivatives, before cells were re-clustered based on the remaining gene modules. We used the expression of known markers to assign cell states. Placodal cells were

then subset and re-clustered using a new set of gene modules identified from the subset cell population. Three main cell states were identified as otic, epibranchial and OEP populations.

Gene expression dynamics at the otic-epibranchial branching point

To model the transcriptional dynamics at the otic-epibranchial branching point, we ordered cells along pseudotime using Monocle2⁵⁶. The lineage tree was rooted to the earliest cell state (ss8-9) which was comprised mostly of OEPs. The expression of genes along the bifurcation point was then modelled using branch expression analysis modelling (BEAM).

To assess whether individual OEPs simultaneously express markers associated with the otic or epibranchial state, we calculated the co-expression of key otic and epibranchial genes within each of the Monocle branches. First, gene expression was binarized based on the presence or absence of gene expression; then the proportion of cells co-expressing pairs of otic-epibranchial genes within each of the branches was calculated. A Kruskal Wallis test was then used to compare the proportion of cells co-expressing otic and epibranchial genes between the three branches. Post-hoc pairwise comparisons between the OEP branch and the otic or epibranchial branch were carried out using two-tailed Wilcoxon rank sum tests.

RNA velocity provides an alternative to Monocle2 for inferring future cell state of individual cells. Spliced, un-spliced and spanning matrices obtained from Velocity²⁰ were subset in R based on genes and cells used to generate the Monocle trajectory. RNA velocity was then calculated using the Velocity.R package, where spanning reads were used to fit gene offsets. Single cell velocities as well as vector fields were subsequently visualised on both tSNE and Monocle DDRTree embeddings.

ChIP-seq and ATAC-seq alignment and peak calling

ChIP-seq and ATAC-seq data were processed and aligned to GalGal6 using the default NF-core ChIP-seq (v1.2.0) and NF-core ATAC-seq (v1.2.0) pipelines⁵⁴, respectively. Reads were aligned using the Burrows-Wheeler aligner. MACS v2.2.7.1 was used to call broad peaks (FDR < 0.1) and narrow peaks (FDR < 0.05) for ChIP-seq and ATAC-seq data, respectively.

Enhancer discovery

Putative enhancers were identified using a custom Nextflow DSL2 pipeline. First, bedtools (v2.29.2) was used to subset ATAC-seq narrow peaks, which overlap with a broad H3K27ac peak, whilst removing those overlapping with broad H3K27me3 peaks. Remaining ATAC-seq peaks were then annotated with Homer (v4.11), using a GalGal6 GTF which was filtered for protein coding genes. Promoter and exonic peaks were then removed. Motif enrichment and functional enrichment analysis were carried out using Homer and g:Profiler, respectively.

Data availability

All data have been deposited in Gene Expression Omnibus (GEO; Accession number GSE168089). Nextflow pipelines and downstream analysis, can be found at <https://github.com/alexthiery/otic-reprogramming>. Full detailed documentation is available at <https://alexthiery.github.io/otic-reprogramming/>. A custom Docker container used can be found at https://hub.docker.com/repository/docker/alexthiery/otic-reprogramming-r_analysis.

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Competing interests. The authors declare no competing interests.

Additional information

Supplementary information is available for this paper.

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