RESEARCH ARTICLE

A systems-level approach reveals new gene regulatory modules in the developing ear

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ABSTRACT
The inner ear is a complex vertebrate sense organ, yet it arises from a simple epithelium, the otic placode. Specification towards otic fate requires diverse signals and transcriptional inputs that act sequentially and/or in parallel. Using the chick embryo, we uncover novel genes in the gene regulatory network underlying otic commitment and reveal dynamic changes in gene expression. Functional analysis of selected transcription factors reveals the genetic hierarchy underlying the transition from progenitor to committed precursor, integrating known and novel molecular players. Our results not only characterize the otic transcriptome in unprecedented detail, but also identify new gene interactions responsible for inner ear development and for the segregation of the otic lineage from epibranchial progenitors. By recapitulating the embryonic programme, the genes and genetic sub-circuits discovered here might be useful for reprogramming naïve cells towards otic identity to restore hearing loss.

KEY WORDS: Auditory system, Cell fate, Chick, Embryo, Hearing, Placode, Transcription factor

INTRODUCTION
In vertebrates, the entire inner ear arises from the otic placode, a simple epithelium next to the hindbrain, which invaginates to form the otic vesicle. The vesicle undergoes extensive morphogenesis, ultimately giving rise to the adult inner ear, an organ of exquisite complexity comprising distinct sensory, non-sensory and neuronal cell types of the auditory and vestibular apparatus. In humans, congenital hearing defects are often due to mutations in cell types of the auditory and vestibular apparatus. In humans, congenital hearing defects are often due to mutations in the otic and epibranchial fates.

Specification towards otic fate occurs early in development and requires diverse signals and transcriptional inputs that act sequentially and/or in parallel. This process is initiated when sensory precursors in the pre-placodal region (PPR) become specified as otic-epibranchial and/or in parallel. This process is initiated when sensory precursors in the pre-placodal region (PPR) become specified as otic-epibranchial progenitors (OEPs) under the influence of fibroblast growth factor (FGF) signalling (Fig. 1A; Ladher et al., 2000; Maroon et al., 2002; Martin and Groves, 2006; Nechiporuk et al., 2007; Nikaido et al., 2007; Phillips et al., 2001; Sun et al., 2007; Umess et al., 2010; Wright and Mansour, 2003). The OEP state is characterized by transcription factors like Foxl1/3 (Khatri et al., 2014;Ohyama and Groves, 2004; Solomon et al., 2003), Dlx genes (Brown et al., 2005; Solomon and Fritz, 2002), Pax2/8 (Christophorou et al., 2010; Freter et al., 2012; Hans et al., 2004; Mackereth et al., 2005) and Spalt4 (Barembaum and Bronner-Fraser, 2007, 2010; Schlosser, 2006). After this step, Wnt and Notch pathways cooperate to promote otic and repress epibranchial character (Fig. 1B; Freter et al., 2008; Jayasena et al., 2008; Park and Saint-Jeannet, 2008; Shida et al., 2015). However, the transcriptional networks that control each step and the distinct differentiation programmes for otic and epibranchial cells are very poorly understood.

Here, we examine the active transcriptome of the developing ear from sensory progenitor to the overtly recognizable placode stage. Major changes occur as cells transit from a progenitor state to become OEPs, highlighting this as the most crucial step during otic induction. Time course analysis reveals previously unknown steps of otic commitment, defined by unique sets of transcription factors, and functional analysis not only reveals new downstream targets of known otic transcription factors, but also allows us to construct the first otic gene regulatory network (GRN) and predict connections therein. Its hierarchical organization reveals how, starting from a few factors initiated by otic induction, information is propagated through the network using positive feedback and feed-forward loops to stabilize otic identity and generate diversity by segregating otic and epibranchial fates.

RESULTS
New genes in otic placode development
The progressive commitment of ectodermal cells towards otic identity occurs gradually, via a series of regulatory interactions that are not well understood. In avian embryos, otic specification begins around the 5-somite stage (ss) and by the 10ss, the otic ectoderm is already committed to its fate and to form an otic vesicle (Adam et al., 1998; Groves and Bronner-Fraser, 2000). To examine the steps leading up to this cell fate decision, we chose three time points for genome-wide transcriptome analysis, corresponding to the stages when (1) cells become specified as OEPs (5-6ss; Fig. 1A), (2) the placode acquires its characteristic thickened morphology (8-9ss) and (3) cells become committed to an otic fate (11-12ss; Fig. 1B).

To identify otic-enriched genes, we compared the otic transcriptome with that of whole embryos (3ss). Several hundred genes are enriched more than 1.5-fold at each stage (5-6ss: 1202 transcripts; 8-9ss: 1079 transcripts; 11-12ss: 1315 transcripts; Fig. 1C,D; Table S2). This analysis recovers many known otic transcription factors (23/27; e.g. Pax2, Gat3, Gbx2, Foxg1, Eyal1 and Soho1; Fig. S1A; Fig. S2). Functional annotation of otic-
enriched transcripts (Fig. 1F-H) reveals progressive commitment to otic identity: ‘epithelium development’ is the most represented gene ontology (GO) term in OEPs (5-6ss; Fig. 1F), but this rapidly changes at 8-9ss and 11-12ss, when ‘inner ear development’ becomes the prominent term (Fig. 1G,H). Interrogating disease association databases reveals that transcripts enriched at the commitment stage are associated with hearing loss (Fig. 1E).

In total, this analysis identified 135 potential transcriptional regulators, of which 112 are novel with respect to the otic placode. To verify that they do represent otic-enriched transcripts, we assessed their expression using complementary methods. Twenty-three factors are indeed expressed in the otic placode according to the gene expression database GEISHA (http://geisha.arizona.edu/geisha/; Fig. S2). We found ten additional factors enriched in placode tissue as assessed by qPCR from dissected otic-epibranchial domains (Fig. S1B,C). Likewise, of 52 transcripts tested by NanoString 46 are present in the otic placode at 11-12ss with mean count >300 (Fig. S1D). For further validation, we performed in situ hybridization of 39 additional factors (Fig. 2; Figs S2, S3). This confirmed that the majority (34/39) is present, although not necessarily restricted to the otic placode. In summary, the transcriptome analysis identifies many genes not previously associated with ear development.

Dynamic changes of gene expression in the developing otic placode
To capture changes in gene expression as otic cells mature, we used complementary approaches: in situ hybridization, transcriptome changes over time and hierarchical clustering. This allowed us to define synexpression groups and uncover distinct transcriptional states during otic placode maturation.

Analysis of transcription factor expression by in situ hybridization
*Lmx1a, Sox13 and Zfbh16* are expressed in OEPs at 5-6ss and their expression persists in the otic placode until at least 12ss (Fig. 2A-F; Fig. S2). In contrast, *Zfhx3, Rere* and *Tcf4* (*Tcf7l2*) only become...
prominent in the otic territory from 10ss onwards (Fig. 2G-L; other factors: Arid3, Atn1, Bach2, Klf8, Prep2, Tead3, Znf384; Fig. S3LJ, O-AD; Fig. S2), whereas Nr2f2, Vgll2 and Klf7 are confined to the epibranchial region (Fig. 2O-R; Fig. S3KL). Prdm1 and Tfap2e transcripts change rapidly: they are broadly expressed at 5ss but then become restricted to the epibranchial territory after 9ss with Tfap2e also present in neural crest cells (Fig. 2M,N; Fig. S3MN). In addition, several transcriptional regulators surround the otic placode at 11-13ss (Fig. S3AE-AJ), whereas others are widely expressed in the ectoderm including the otic territory (Fig. S3A-H; Fig. S2) or also present in neural crest cells (Fig. S3AJ-AN). We summarize the temporal and spatial expression of 95 known and new transcripts in Fig. S2.

**Hierarchical clustering identifies distinct TF synexpression groups during otic commitment**

Hierarchical clustering of all transcription factors that are either enriched in the otic placode compared with the whole embryo (Fig. 1) or differentially expressed over time (Fig. 3) reveals five major clusters denominated transcription factor cluster 1-5 (TFC1-5; Fig. 4A; Table S4). These clusters show distinct temporal profiles (Fig. 4A-F) and generally confirm our analysis reveals distinct regulatory states as cells acquire otic identity. Substantial transcriptome rearrangements occur within a brief period of only 6-8 h (from 1ss to 5ss) during the first step of otic induction: anterior character is inhibited and cells are specified around 10ss together with Fig. 2), whereas a small group of transcripts [Irx5, Lmx1a, cMyb (Betancur et al., 2011), Prdm1, Sall4 (Barembaum and Bronner-Fraser, 2007), Sox13, Zbtb16 and Znf385c] becomes upregulated together with Fox2 and Etv4 (Figs 2, 4; Figs S2, S3). Sox10 expression is initiated around 10ss together with Foxg1 and Dlx3 (Betancur et al., 2011; Khudyakov and Bronner-Fraser, 2009; Yang et al., 2013), and Prdm1 becomes restricted to the epibranchial territory (Fig. 2), where it is co-expressed with Fox2, Foxi2 and Sox3 (Abu-Elmagd et al., 2001; Freter et al., 2008; Groves and Bronner-Fraser, 2000). Thus, already at the 10ss stage, otic and epibranchial progenitors begin to segregate and become molecularly distinct. As cells become committed to otic identity many new transcription factors start to be expressed (Figs 2, 4; Figs S2, S3) and the otic and epibranchial fates continue to diverge. In summary, our time course analysis reveals distinct regulatory states as cells acquire otic identity. Substantial transcriptome rearrangements occur within a brief period of only 6-8 h (from 1ss to 5ss) during the first step of otic induction: anterior character is inhibited and cells are specified...
as OEPs. Our analysis defines new factors that characterize a transcriptional state characteristic for OEPs at 4-5ss. As development proceeds, known ear-specific transcripts become more prominent, as do genes associated with hearing impairment, suggesting that our data might harbour new candidate deafness genes.

Pathway analysis suggests potential novel regulators of otic placode formation

Signals from the surrounding tissues induce and pattern the otic placode. Although the role of FGF, Notch and Wnt pathways is well established (Abello et al., 2010; Freter et al., 2008; Ladher et al., 2000; Ohyama et al., 2006; Park and Saint-Jeannet, 2008; Phillips et al., 2004; Urness et al., 2010), it is likely that other signals are also involved. To explore this possibility, we used hierarchical clustering of all otic-enriched genes (from Fig. 1) together with differentially expressed genes from stage-wise comparisons (from Fig. 3) to generate six major clusters (denominated C1-C6; Fig. S4).

First, we evaluated pathways known to mediate otic development. As expected, Notch signalling components are present throughout placode formation and are over-represented in OEPs and in the placode (Fig. 3C; Fig. S4A,D) with Lfng expression increasing sharply as the placode forms and Deltex2 and -4 rising gradually. Wnt signalling components are highly enriched in cluster C2 (Fig. S4A,C) with the Wnt receptors Fzd1-3 and mediators Lef1 and Tcf7l2 increasing sharply. In contrast, the Wnt antagonist Sfrp2 drops sharply at 5-6ss. Components of the non-canonical Wnt pathway, such as Wnt5a, rise gradually together with Rac1 and Jun, suggesting a role in placode assembly and morphogenesis. These findings are consistent with known changes in signalling events during otic commitment and therefore confirm the usefulness of this
Next, we investigated whether new pathways emerge from this analysis. As OEPs become specified, components of the steroid biosynthesis pathway are markedly upregulated (Fig. 3E,G), whereas TGFβ signalling components drop sharply (Fig. 3I,J). Spliceosome components (Fig. S4A,B, cluster C2) peak at 5-6ss and 8-9ss. Consistent with this, spliceosomal defects are known to cause craniofacial disorders, some of which are associated with hearing loss (Lehalle et al., 2015). As a morphological placode forms, focal adhesion-related components become increasingly enriched, suggesting a role in placode assembly (Fig. 3E,F). These observations point to signals and pathways not previously associated with ear formation to explore in the future.

Fig. 4. Clusters of otic transcription factors. (A) Otic transcription factors from the enrichment and time course analysis cluster into five clusters (TFC1-5) based on the row z-score of fold change relative to the PPR at 0ss. (B-F) Expression level of the top 50% transcription factors in each cluster. Line in the top right of each cluster represents the overall expression profiles across the three time points. See also Table S4.
Regulatory relationships reveal distinct transcriptional modules during otic commitment

Our time course analysis of gene expression predicts a transcriptional hierarchy during otic induction. To begin to test this hierarchy, we selected three transcription factors, Etv4, Pax2, and Lmx1a, for perturbation experiments for the following reasons. The transition from sensory progenitors to OEPs is mediated by the GFG pathway (Ladher et al., 2000; Maroon et al., 2002; Martin and Groves, 2006; Nechiporuk et al., 2007; Nikaio et al., 2007; Phillips et al., 2001; Sun et al., 2007; Urness et al., 2010; Wright and Mansour, 2003). Accordingly, the GFG mediator Etv4 is expressed in OEPs (Lunn et al., 2007) and upregulated 7.5-fold from 1ss to 5-6ss (Table S3). Only ten transcription factors are strongly initiated at OEP stages (6- to 235-fold), with Pax2 being the top factor (235-fold; Table S3), but Lmx1a is one of the few genes (6.3-fold; Table S3) exclusively expressed in otic, but not epibranchial cells (Fig. 2A,B). To explore the gene network downstream of these factors, we knocked down their expression by electroporation of antisense morpholino oligonucleotides at 1-2ss (MOs; Barembaum and Bronner-Fraser, 2010; Betancur et al., 2011; Christophorou et al., 2010). Experiments were assessed by NanoString nCounter at 10-12ss using a total of 216 probes including 70 otic genes (mostly transcription factors), markers for placode progenitors, other placodes, the neural plate, neural crest cells and non-neural ectoderm. This analysis provides a large-scale view of transcriptional changes in a single experiment enriching previously published data, which generally assessed a few genes at a time. In addition, selected transcripts were also assessed by RT-qPCR and/or in situ hybridization (Fig. 7; Fig. S6; Table S5). A gene was considered to be activated or repressed when its expression was reduced or enhanced after knockdown, respectively [NanoString: normalized mean count >300, ±1.2-fold change, P-value (P-adj) =<0.1; RT-qPCR: ±1.5-fold change, P=0.05; in situ hybridization: absence or reduction of signal in electroporated cells]. These data allow us to add functional links between Etv4, Pax2, Lmx1a and other transcription factors in the otic GRN (Fig. 6), although cis-regulatory analysis will be required to distinguish between direct and indirect interactions. Although our experiments do not determine precisely when these interactions take place, we can infer this from our expression data, which show the onset of target genes.

Etv4 and Pax2 control the onset of OEP factors

Etv4 knockdown leads to a reduction of Pax2 expression (Fig. 5B-B′; Fig. S6A,B) confirming a requirement of GFG activity for Pax2 expression. In addition, Etv4 activates other early OEP transcripts (Irx5, Prdm1, Zbth16, Sall4, Sox8; Fig. 5A-F; Barembaum and Bronner-Fraser, 2007; Yang et al., 2013), and is also required for genes present at placode stages (Lef1, Lmx1b, Sox10, Tcf4; Table S5). In contrast, Etv4 represses some PPR genes (Six1, Eya2), the OEP factor Znfl35ac and late otic placode transcripts (Tead3, Arid3, Sall1; Fig. S6A,B; Table S5).

Many Etv4 targets are also regulated by Pax2 (OEP transcripts: Irx5, Prdm1, Zbth16, Sall4; placode genes: Lef1, Lmx1b, Sox10, Tcf4; Fig. 5G-J″; Fig. S6A,C; Table S5). In addition, Pax2 activates the Etv4-independent OEP genes Lmx1a (Fig. 5H-I′) and Sox13, the placode transcripts Eya1, Meis1, Zfhx3 and Znf521 and maintains the PPR factor Six1, while repressing the posterior PPR genes Foxi3 and Gbx2, which are normally cleared from the placode as it matures, the trigeminal marker Pax3 (Wakamatsu, 2011), late onset otic genes (Foxg1, Dlx3) and Klf7, which is later expressed in the epibranchial region (Fig. S6A,C). Pax2 is also required for the epibranchial-specific factor Vgll2 (Fig. 5K-K′). Electroporation of control morpholinos does not affect otic gene expression (Fig. 5N-P′). Thus, many inner ear transcription factors depend on Etv4 and/or Pax2 activity placing these factors at the top of the otic hierarchy.

Lmx1a and Pax2: a positive feedback loop that maintains OEP factors and represses alternative fates?

Exploring Lmx1a function, we find that Pax2 depends on Lmx1a input. Pax2 expression is reduced when Lmx1a is knocked down (Fig. 5L-M′; Fig. S6A). Thus, they mutually regulate each other in a positive feedback loop and have common targets: both are required for Sox13 and Zbth16 expression (Fig. 6A). In addition, Lmx1a is necessary for Foxg1 and Gbx2 expression. Like Pax2, Lmx1a also suppresses several transcripts (Fig. 5L; Fig. 6A), among them the PPR genes Six1 and Foxi3 and the lens/olfactory factor Pax6. Thus, together both transcription factors appear to promote OEP, but might also participate in the repression of alternative fates.

DISCUSSION

Commitment to otic fate is initiated by the specification of OEPs from the posterior part of the pre-placodal region, followed by the acquisition of columnar placode morphology and, finally, placode invagination to form the otic vesicle. During this process, the otic territory is exposed to signals from surrounding tissues, and as morphogenetic events shape the embryo, its extrinsic environment changes constantly. As a result, ectodermal cells first initiate a transcriptional programme unique to otic progenitors and then form a patterned otic vesicle. Here, we have greatly expanded this transcriptional repertoire by identifying more than 100 factors that might be crucial for placode development, and are also new candidate genes for hearing impairment. Exploring the dynamic changes in gene expression over time together with perturbation analysis of selected transcription factors and integrating data from the literature (Fig. S5) allows us to propose the first GRN for otic commitment.

A transcriptional mechanism for OEP specification

To establish the first GRN that describes how sensory progenitor cells are committed to the inner ear lineage, we used a strategy that measures changes of all otic transcription factors after experimental perturbation using partial knockdown of three transcription factors (Fig. 7). This GRN has the deep structure characteristic of embryonic networks (Davidson, 2010), revealing the hierarchical organization of the process that drives otic commitment. Within this network, distinct transcriptional modules can be identified that gradually establish otic identity.

The posterior PPR module

For the otic placode to develop, ectodermal cells must first go through a PPR state (Martin and Groves, 2006), which is identified by Six and Eya family members and by Irx1 and Dlx5/6. Six1 is an important PPR determinant (Ahrens and Schlosser, 2005; Brugmann et al., 2004; Christophorou et al., 2009) and Irx1, Dlx5 and Foxi3 are known to regulate its expression (Glavic et al., 2004; Khatri et al., 2014; Pieper et al., 2012; Sato et al., 2010; Woda et al., 2003). In the posterior PPR, Six1, Eya2, Gbx2 and Foxi3 are crucial for otic placode formation (Brugmann et al., 2004; Christophorou et al., 2009; Solomon et al., 2003; Steventon et al., 2012). Gbx2 restricts the expression of Otx2 to the anterior PPR (Steventon et al., 2012), whereas Foxi1/3 and the Six1/Eya2 complex regulate each other in a positive feedback loop (Khatri et al., 2014), perhaps to maintain posterior PPR identity. Together, all four proteins provide...
crucial input for the OEP transcription factor Pax2 (Christophorou et al., 2010; Hans et al., 2004, 2007; Solomon et al., 2003, 2012): loss of Foxi1 function in fish and repression of Gbx2 and Six1 targets genes in *Xenopus* and chick, respectively, lead to the absence of Pax2 (Fig. 6B; Fig. 7A). However, none of these transcription factors is sufficient to induce Pax2 in non-otic cells, suggesting that additional input is required.

### The OEP module

It is well established that FGF signalling induces OEPs from the posterior PPR (Ladher et al., 2000, 2005; Maroon et al., 2002; Martin and Groves, 2006; Nechiporuk et al., 2007; Nikaido et al., 2007; Philips et al., 2001; Sun et al., 2007; Urness et al., 2010) and we show that the FGF target Etv4 is crucial for this process. *En-4* is upregulated as posterior PPR cells transit to OEPs and is required for
Pax2 expression. Thus, a typical ‘AND’ gate controls Pax2 in OEPs: its expression requires dual input from the posterior PPR factors Six1, Foxi3 and Gbx2 and from the FGF mediator Etv4 (Fig. 6B; Fig. 7A).

Our data suggest that Etv4 and Pax2 work in concert to promote otic identity and place them at the top of the transcriptional hierarchy for otic commitment (Fig. 7A). Together, they rapidly activate an OEP module consisting of Sox8, Lmx1a, Zbtb16, Sox13, Prdm1, Sall4, Znf385c and Irx5. The OEP module also contains the Pax2- and Etv4-independent factor cMyb (Betancur et al., 2011); however, its upstream regulators are currently unknown. As Etv4 and Pax2 share many targets, two scenarios could explain their mode of action. Etv4 might only regulate Pax2, which in turn controls other targets in a linear hierarchy (Fig. 6C; for simplicity, we depict this possibility in Fig. 7). However, it is equally possible that Etv4 and Pax2 act in a feed-forward loop, in which Etv4 is required for Pax2 and both together control the expression of downstream targets (Fig. 6D) as is indeed the case for Sall4 (Barembaum and Bronner-Fraser, 2007).

Although these and previous data implicate Pax2 as a key factor for otic specification and proliferation in chick (Christophorou et al., 2010; Freter et al., 2012), the mouse otic placode still forms in the absence of Pax2 (Burton et al., 2004; Favor et al., 1996; Torres et al., 1996) or Pax2 and Pax8 function (Bouchard et al., 2010). The fact that sauropsids have lost the Pax8 gene (Christophorou et al., 2010; Freter et al., 2012) could explain the more prominent role of Pax2 in the otic GRN.

The newly identified OEP factors might play an important role in ‘locking’ cells in an OEP transcriptional state, where they are competent to respond to signals committing them towards otic and epibranchial fate. It has previously been suggested that continued FGF activity inhibits otic placode formation, while promoting epibranchial cells (Freter et al., 2008). Indeed, when chick OEPs are cultured in isolation they maintain otic identity from 5-6ss onwards and even generate neurons in the absence of additional signalling (Adamska et al., 2001; Groves and Bronner-Fraser, 2000). These findings suggest that in an OEP state ear precursors are FGF independent and we propose that the OEP module could be instrumental to maintain cell identity. Like Pax2 and Lmx1a, other OEP transcription factors might act in positive feedback loops to maintain OEP gene expression and repress alternative fates.

Two distinct steps segregate otic and epibranchial progenitors

Our temporal analysis reveals two steps during the segregation of otic and epibranchial fates (Fig. 7A,B). Under continued FGF signalling, OEPs differentiate into epibranchial cells (Freter et al., 2008; Sun et al., 2007). This is consistent with our finding that Etv4 is required for epibranchial transcripts, as is Pax2. Dependent on the cellular context, Pax2 is likely to use different partners to impart cell fate (Kamachi and Kondoh, 2013; Stolt and Wegner, 2010): SoxE group members are prominent in otic cells, but they are absent from epibranchial placodes, where SoxB group members could represent major Pax2 partners (Ishii et al., 2001; Wood and Episkopou, 1999). In the otic lineage, expression of a small set of transcription factors (Sox10, Foxg1 and Dlx3; Fig. 7B) is initiated downstream of Pax2 and the OEP module; for example, the Sox10 enhancer is directly controlled by Pax2, Sox8 and cMyb (Betancur et al., 2011), whereas epibranchial cells begin to initiate a different transcriptional programme.

In a second step, a large number of otic transcripts begin to be expressed, among them several that depend on canonical Wnt
Fig. 7. Gene regulatory network incorporating new functional data. (A-C) Signalling inputs, gene expression changes and regulatory relationships at the three different stages. Regulator links are based on data from the literature (Fig. S5) and our perturbation experiments (Fig. 5; Fig. S6A-C). Direct interactions confirmed from literature are indicated with blue diamonds and bold lines. As enhancers for most genes are currently unknown, the network assumes the simplest interactions depending on perturbation data (see also Fig. 6). Epi, epibranchial domain; OEPD, otic-epibranchial progenitor domain; OEPs, otic epibranchial progenitors; OP, otic placode; pEpi, pre-epibranchial domain.
signalling from the neural tube (Freter et al., 2008; Ohyama et al., 2006) (Fig. 7C). Interestingly, FGF signalling, either directly or indirectly, promotes the Wnt mediators Lef1 and Tcf4, suggesting that these upstream events might prime OEP cells for Wnt signalling. In contrast, under the influence of bone morphogenetic protein (BMP) signalling (Begbie et al., 1999) epibranchial progenitors continue to diverge from otic cells and the two fates become firmly established. In summary, the temporal resolution of our analysis highlights the complexity of cell fate decisions and reveals new transcriptional states as sensory progenitor acquire ear and epibranchial identity.

The OEP module: a molecular circuit re-deployed in other organs?

Our analysis has defined a new transcriptional circuit, operating downstream of Pax2 and FGF/Etv4, which we propose is important for the specification of otic-epibranchial precursors. In addition to the ear, members of the OEP module are also co-expressed in the developing kidney and limb, including Zbtb16 (Cook et al., 1995), Lmx1 (Fernandez-Teran et al., 1997), Prdm1 (Ha and Riddle, 2003) as well as Sox8 (Chimal-Monroy et al., 2003) and Sox13 (Kido et al., 1998; Wang et al., 2006). Members of the Six and Eya families lie upstream of the OEP module, and together with Pax proteins are part of the fly retinal determination network (Salzer and Kumar, 2009), which in vertebrates also regulates the formation of other organs including the ear and kidney. Interestingly, the homologues of Zbtb16, Lmx1a and Prdm1 participate in fly eye signalling. In contrast, under the influence of bone morphogenetic protein (BMP) signalling (Begbie et al., 1999) epibranchial progenitors continue to diverge from otic cells and the two fates become firmly established. In summary, the temporal resolution of our analysis highlights the complexity of cell fate decisions and reveals new transcriptional states as sensory progenitor acquire ear and epibranchial identity.

Uncovering new candidate deafness genes

Although much progress has been made recently to identify the genetic causes for hearing impairment in humans, many causative genes remain to be uncovered. In particular, mutations in developmental genes associated with human deafness, for example Six1 or Eya1 mutations are known to cause Branchio-Otorenal Syndrome, an autosomal dominant disorder (Ruf et al., 2004). Functional annotation of our transcriptome data reveals a significant association with hearing loss, in particular for transcripts enriched in the committed otic placode (11-12ss, Fig. 1E). Indeed, the number of otic placode-enriched genes that fall into known human deafness loci is significantly higher than expected for a set of random genes (data not shown). These findings suggest that our data might harbour a number of novel candidates for congenital malformations of the ear and for hearing impairment.

In summary, integrating data from the literature (Fig. S5) and our new analysis has allowed us to establish the first gene regulatory network (Fig. 7) that models the early stages of ear development. By identifying key otic genes and the linkages between transcription factors at different levels of the network, we define new distinct regulatory states as cells acquire otic identity as well as the regulatory loops that stabilize cell fate decisions. In the future it will be important to identify the cis-regulatory elements controlling otic gene expression to determine direct and indirect interactions. The information gleaned from our analysis of otic transcriptional programmes will be crucial for future experiments aiming to reprogramme cells after loss of hearing and/or balance for the purpose of repair and regeneration.

**MATERIALS AND METHODS**

**Tissue dissection and embryo experiments**

Fertilized hens’ eggs (Winter Farm, Herts) were incubated in a humidified incubator at 38°C to reach the appropriate stage. For dissection, embryos were isolated from the egg and pinned out ventral side up on a resin plate in Tyrode’s saline. The endoderm and mesoderm layers were removed using a G-31 syringe needle in the presence of a small volume of 10 mg/ml dispase before dissecting the future otic ectoderm based on the region expressing Pax2 (Streit, 2002), rostral to the first somite and adjacent to future hindbrain rhombomeres 4–6.

For *in situ* hybridization, embryos were isolated in PBS, fixed in 4% paraformaldehyde in PBS and processed as described previously (Streit and Stem, 2001). Digoxigenin-labelled antisense RNA probes were synthesized with T7, T3 or SP6 RNA polymerase (Roche) as appropriate from expressed sequence tags, cloned fragments or previously published plasmids (Table S6).

For morpholino knockdown experiments, electroporation at 0-1ss was performed either unilaterally with control and experimental morpholino, or in the same embryo with control and experimental morpholino on different sides. Pax2 and Etv4 morpholinos were validated previously (Betancur et al., 2011; Christopoulos et al., 2010); as controls, standard control morpholinos (5′-CCTCTTTACCTCGATCAATTTATA-3′) were used. For Lmx1a, a splicing-blocking morpholino (5′-ACCCCACTGTCCCCC-ATACCTTTCC-3′) targeting the exon 4-intron 4 junction was used and deletion of exon 4 was confirmed by RT-PCR followed by sequencing the PCR product. Changes in gene expression were assessed by whole-mount *in situ* hybridization or from five to ten dissected otic placodes by RT-qPCR or NanoString. For dissection, the non-electroporated or control morpholino electroporated side and the first somite is used as a guide.

**RNA isolation, library construction and sequencing**

About 100 placodes were collected from each stage for RNAseq library preparation. Immediately after dissection, tissues were lysed in lysis buffer (Ambion) and RNA was isolated using the RNAqueous-Micro Kit (Ambion). Libraries were prepared with TrueSeq RNA Sample Preparation V2 kit (Illumina) and sequenced with Illumina HiSeq 2000 (Illumina) to 1×50 cycles or HiSeq 2500 to 2×100 cycles.

**Sequence alignment**

Reads were aligned with TopHat2 (v2.0.7) to Ensembl chick genome Galgal4.71 (Kim et al., 2013). Transcripts for individual samples were assembled with Cufflinks (v2.1.1) (Trapnell et al., 2010), with combined Ensembl gene annotation (Galgal4.71.gtf) and Refseq acquired from UCSC table browser as a guide. All assemblies were merged to obtain a merged annotation file, which was passed to CuffDif (v2.1.1) to obtain normalized RPKM value for each gene, and to easyRNAseq (v2.1.0) to retrieve the count table for all genes (Dellhomme et al., 2012). All sequencing data were deposited in Gene Expression Omnibus (GEO) under accession number GSE69185.

**Identification and analysis of differentially expressed genes**

DEseq (v1.16.0) was used to identify enriched otic genes relative to the whole embryo (Anders and Huber, 2010) based on the count table generated above. A gene was considered to be expressed in the otic region when the normalized RPKM was >4, and the number of normalized counts was >300. Owing to a lack of biological replicates, ’Blind’ mode was used in DEseq, which treats the two samples to be compared as replicates to estimate the variance of gene expression. This approach assumes that most genes are not differentially expressed (as would be the case in biological replicates), thus generally overestimating variance, because the variance among samples from different conditions is usually larger than among biological replicates. Therefore, this method produces very conservative results with small numbers of differentially expressed genes (Anders and Huber, 2010). Using F-adj<0.1 and a fold change of >1.5, only a fraction of known otic genes (14/37) is recovered (Table S1). To maximize the discovery of otic
placode-enriched genes, all genes with a fold change of >1.5 were included as candidates for further analysis. This analysis recovers 27/37 known otic genes. Validation by in situ hybridization was used as a secondary screen to ensure that the gene list did indeed represent otic placode-enriched transcripts. Indeed, of all 39 factors screened by in situ hybridization only five are not expressed in the placode, validating this as a useful strategy to identify genes enriched in otic cells.

Gene ontology analysis for differentially expressed genes was performed with DAVID (DAVID Bioinformatics Resources 6.7, http://david.abcc.ncifcrf.gov/) (Huang da et al., 2009a,b). Disease-related enrichment was analysed with Webgestalt (Wang et al., 2013; Zhang et al., 2005).

**Partitioning of otic genes into different clusters**
Both enriched otic genes and differentially expressed transcription factors from pairwise analysis (except downregulated genes at 5-ss6 relative to 0ss) were used for hierarchical cluster analysis. Fold change of these factors at 5-ss6, 8-ss9 and 11-12ss relative to 0ss was transformed into row z-score with heatmap.2 and corresponding heatmaps were generated using gplot within R (R Core Team, 2012; Warnes, 2012). Otic genes were clustered in the same way to generate the clusters in Fig. S4.

**Quantitative RT-PCR and NanoString nCounter analysis**
RNA from dissected otic tissue and the whole embryo was isolated using the RNAqueous-Micro Kit (Ambion) and reverse transcribed. Primers for target genes were designed with PrimerQuest (IDT). qPCR was performed in technical triplicates using Rotor-Gene Q (Qiagen) with SYBR green master mix (Roche). The ΔΔCt method was used to calculate the fold change, which was expressed as FC=2^ΔΔCt (Livak and Schmittgen, 2001). Gapdh, Hprt and Rplp0 were used as reference genes to calculate the fold change. RT-qPCR to validate the otic-enriched genes was performed from a single biological replicate, and RT-qPCR for morpholino experiments used two biological replicates; the P-value generated from a two-tailed Student’s t-test was used to evaluate the statistical significance.

NanoString analysis was performed in triplicate per experimental condition with the nCounter Analysis System using a customized probe set of 216 genes. Five to ten tissues were lysed in lysis buffer (Ambion) and was used to evaluate the statistical significance.

To establish links between upstream regulators and their downstream targets, unless otherwise stated we assumed the most parsimonious pathway: e.g. if gene 1 regulates gene 2 and 3, and gene 2 regulates gene 3 we assumed the simplest explanation of gene 1 gene 2 gene 3.

**Acknowledgements**
We thank Ewa Kolano, Annabelle Scott, Chia-Li Liao and Hilary McPhail for excellent technical assistance and C. D. Stern for critical reading of the manuscript.

**Competing interests**
The authors declare no competing or financial interests.

**Author contributions**
A.S. designed the experiments; J.C. and M.T. collected otic tissues; R.R. performed RNAseq for PPR tissue; J.C. analysed RNAseq data; J.C. and M.T. performed most functional experiments and analysed all data together with A.S. M.B. contributed to the knockdown experiments; M.S.-C. contributed to RNAseq experiments; J.C., A.S. and M.E.B. wrote the manuscript.

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**Data availability**
All sequencing data have been deposited in Gene Expression Omnibus under accession number GSE69185 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi? acc=GSE69185).

**Supplementary information**
Supplementary information available online at http://dev.biologists.org/lookup/doi/10.1242/dev.148494.supplemental

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**References**
Adamska, M., Herbrand, H., Adamski, M., Krüger, M., Braun, T. and Bober, E. (2001). FGFs control the patterning of the inner ear but are not able to induce the full ear program. Mech. Dev. 109, 303-313.


Transcription factors enriched in the otic placode

A

log2 fold change

mean of normalized counts

2×10^5
5×10^3
t=0.02
5-6ss
76 TFs
Eya1
FoxG1
Lmx1A
Pax2
Gata3

8-9ss
72 TFs
Eya1
FoxG1
Irx1
Pax2
Eya2
Gbxa2
Rere

11-12ss
107 TFs
Eya1
FoxG1
Pax2
Sox8
Gata3
Rere

RT-qPCR validation of 10 enriched transcription factors

B

5-6ss
RNAseq
RT-qPCR

Prdm1
Tcf4
Zhix3
Rere

Lmx1b
Lmx1a
Sox13
Zbtb16

11-12ss
RNAseq
RT-qPCR

C

log2 fold change

log2 fold change

Count of enriched genes in Nanostring assay

D

6 genes

Ranked genes
Fig. S1 Otic-enriched transcriptional regulators. A. Transcripts enriched in the otic placode were identified by comparison of the otic transcriptome to the whole embryo at ss3 and known and putative transcription factors were extracted. Numbers of otic enriched transcription factors (TF) at each stage are indicated in the lower right corner of each diagram. A few known otic regulators are labelled. B, C. Validation of enrichment of 10 otic transcription factors by RT-qPCR. At both 5-6ss (B) and 11-12ss (C), fold change was calculated relative to the expression in whole embryo at 3ss. Fold change from RNAseq is plotted alongside for comparison of consistency between two assays. D. Expression of 52 otic genes evaluated by NanoString. The genes are ranked based on their count. 46/52 otic enriched genes pass the count >300 threshold at 11-12ss.
Fig. S2 Summary of the expression patterns of 95 otic genes. This table was assembled using data from the literature and our own new data. References are provided in the table.
**Fig. S3 Expression patterns of new otic and epibranchial transcription factors.** *Arid3a* (A, B), *FoxP4* (C, D), *Hmg1* (E, F), *Irx5* (G, H) and *FoxP1* (I, J) are expressed from OEP stages and remain widely expressed in the otic and epibranchial territories, except for *FoxP4* which becomes confined to the epibranchial region (D). *Klf7* (K, L) is already expressed at 6ss and becomes restricted to the epibranchial domain at 12ss (L). *Tfap2e* (M, N) is expressed in neural crest cells and later in the epibranchial placodes. Late onset genes (after 11-12ss) include *Arid3c* (O, P), *Atnl* (Q, R), *Znf384* (S, T) *Klf8* (U, V), *Prep2* (W, X), *Tead3* (Y, Z), *Bach2* (AA, AB), *Sox11* (AC, AD), *Tbx10* (AE, AF), *Eya4* (AG, AH), *Lef1* (AI, AJ), *Ets1* (AK, AL) and *Dach2* (AM, AN) are localized at the ridge the otic cup and/or in neural crest cells (*Lef1, Ets2*). Most factors are also expressed in other domains like the neural tube and underlying mesoderm.
Fig. S4 Otic genes are partitioned into different clusters. A. 6 clusters are revealed by hierarchical clustering (C1-6). The top enriched biological process and signalling pathway are indicated for each cluster. B-D expression profiles of signalling pathway components in cluster C2 (B), C5 (C) and C6 (D). * in C indicates genes whose expression is plotted against the second axis on the right hand side, while * in D indicates that Log2(RPKM) is used to plot DTX4 expression.
### Interactions within the posterior PPR

<table>
<thead>
<tr>
<th>Source</th>
<th>Interaction</th>
<th>Target</th>
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<th>Evidence</th>
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<td>Dlx5/6 (Dlx3b/4b), Six1/Eya2</td>
<td>Promote</td>
<td>Foxi3 (Foxi1)</td>
<td>Chick, Xenopus, Zebrafish</td>
<td>(Hans et al., 2007; Hans et al., 2004; Khatri et al., 2014; Nissen, 2003; Phillips et al., 2004; Pieper et al., 2012; Solomon, 2003)</td>
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<td>Promotes</td>
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<td>Otx1/2/5</td>
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### Interactions from PPR to OEPs

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<th>(Yang et al., 2013)</th>
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<td>Promote</td>
<td>Pax2</td>
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<td>(Bricaud and Collazo, 2006; Christophorou et al., 2009; Frerer et al., 2008; Hans et al., 2007; Hans et al., 2004; Khatri et al., 2014; Ladher et al., 2000; Ladher et al., 2005; Leger and Brand, 2002; Mackereth et al., 2005; Maroon et al., 2002; Martin and Groves, 2006; Padanad et al., 2012; Padanad and Riley, 2011; Phillips et al., 2004; Solomon and Fritz, 2002; Solomon et al., 2004; Stevenson et al., 2012; Sun et al., 2007; Wright and Mansour, 2003)</td>
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<td>Sall4</td>
<td>Chick</td>
<td>(Barembaum and Bronner-Fraser, 2010)</td>
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### Interaction between mesoderm and neural plate

| FGF | Promotes | Wnt | Chick | (Ladher et al., 2000; Phillips et al., 2004; Rogers et al., 2011) |

*Fig. S5 Known interactions from the literature.

The table summarizes the interactions described in the otic gene regulatory network (Fig.7) including the appropriate references. Gene names are colour-coded as in the networks and use chick nomenclature; names used in other species are shown in brackets. * Experimentally verified direct interactions. References are listed in the final section of supplementary information.
Fig. S6 Summary of perturbation experiments. A. Summary of Etv4, Pax2 and Lmx1a morpholino knockdown experiments evaluated with qPCR, NanoString (NS) and in situ hybridization (ISH). B, C. Evaluation of Pax2 and Etv4 knockdown by qPCR. * significantly affected genes (p-value<0.05).
**Table. S1 Otic-enriched genes.** Genes enriched in the otic placode at 5-6ss, 8-9ss and 11-12ss using fold change > 1.5 and a padj<0.1. Genes labelled “NA” are removed because their RPKM is <4 or count number <300.

Click here to Download Table S1

**Table. S2 Otic-enriched genes.** Genes enriched in the otic placode at 5-6ss, 8-9ss and 11-12ss using fold change >1.5 as cut-off. Genes labelled “NA” are removed because their RPKM is <4 or count number <300.

Click here to Download Table S2

**Table. S3 Time course of differentially expressed genes.** Genes labelled “NA” are removed because RPKM is <4 or count number <300.

Click here to Download Table S3
**Table. S4 Otic transcription factors used for cluster analysis.** The data sheet contains transcription factors enriched in otic territory (see Fig 1) and differentially expressed from time-course analysis (see Fig 3) except those downregulated at 5-6ss relative to HH6. The fold change was calculated with DEseq relative to HH6 and the normalized RPKM was acquired from Cuffdiff2.

Click here to Download Table S4

**Table. S5 Genes affected by Etv4 and Pax2 knockdown.** NanoString nCounter data showing changes in gene expression after Etv4 and Pax2 knockdown. Genes with a mean expression count of less than 300 in either condition are excluded from any analysis.

Click here to Download Table S5
Table S6. Plasmids used for RNA probe synthesis

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Table S6. Continued

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Probes obtained from different sources

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References


