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## Pax2 coordinates epithelial morphogenesis and cell fate in the inner ear

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#### Introduction

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The vertebrate inner ear develops from a simple epithelium, the 41otic placode, next to the hindbrain (Ohyama et al., 2007; Riley and 42Phillips, 2003; Whitfield et al., 2002). The placode invaginates to form 43the otic vesicle, which folds into the complex structure of the mature 44 ear over time. Otic fate is induced by signals from adjacent tissues: 45members of the fibroblast growth factor (FGF) family induce the pre-46 otic field, which is then further refined through a combination of 47Notch and Wnt signalling (Freter et al., 2008; Jayasena et al., 2008; 48 49 Ladher et al., 2000; Leger and Brand, 2002; Maroon et al., 2002; Ohvama et al., 2006; Phillips et al., 2001; Wright and Mansour, 2003). 50In response to these signals, cells begin to express a series of 51transcription factors that successively impart otic identity: among the 5253earliest factors are Foxi1 and Dlx genes, followed Pax8, Pax2 and Sox3 in the otic-epibranchial territory and Eya1, Gata3, Gbx2 and Sox9 in the 54otic region (for review see: (Ohyama et al., 2007; Riley and Phillips, 55562003; Schlosser, 2006); (Ladher et al., 2010). Studies in zebrafish have led to a model where Foxi1 acts upstream of Pax8, while Dlx proteins 57activate Pax2 slightly later and both Pax proteins then cooperate to 5859promote otic fate (Hans et al., 2004; Mackereth et al., 2005; Nissen 60 et al., 2003; Solomon et al., 2003, 2004), but most of these interactions 61 remain to be verified in amniotes. For example, while Pax2 mutant

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### ABSTRACT

Crucial components of the vertebrate eye, ear and nose develop from discrete patches of surface epithelium, 23 called placodes, which fold into spheroids and undergo complex morphogenesis. Little is known about how 24 the changes in cell and tissue shapes are coordinated with the acquisition of cell fates. Here we explore 25 whether these processes are regulated by common transcriptional mechanisms in the developing ear. After 26 specification, inner ear precursors elongate to form the placode, which invaginates and is transformed into 27 the complex structure of the adult ear. We show that the transcription factor Pax2 plays a key role in 28 coordinating otic fate and placode morphogenesis, but appears to regulate each process independently. In 29 the absence of Pax2, otic progenitors not only lose otic marker expression, but also fail to elongate due to the 30 loss of apically localised N-cadherin and N-CAM. In the absence of either N-cadherin or N-CAM otic cells lose 31 apical cell-cell contact and their epithelial shape. While misexpression of Pax2 leads to ectopic activation of 32 both adhesion molecules, it is not sufficient to confer otic identity. These observations suggest that Pax2 33 controls cell shape independently from cell identity and thus acts as coordinator for these processes. 34

mice show late ear defects, the ears of Pax8 mutants develop 62 relatively normally and double mutant phenotypes have not been 63 examined (Burton et al., 2004; Christ et al., 2004; Torres et al., 1996). 64 Thus, in amniotes, a role for Pax genes in otic specification has not 65 been established. 66

Following otic induction ectodermal cells undergo morphological 67 changes critical for the formation of the placode proper and for its 68 subsequent development into a functional ear. Like the neural plate 69 (Colas and Schoenwolf, 2001; Schoenwolf and Franks, 1984; Wallingford, 70 2005), placode cells first lengthen along their apical-basal axis to form a 71 columnar epithelium and invagination is initiated by apical constriction 72 leading to vesicle formation (Bancroft and Bellairs, 1977; Hilfer et al., 73 1989; Schook, 1980a,b). This process is driven by contraction of the 74 apically localised F-actin network by myosin (Sai and Ladher, 2008); for 75 review: (Sawyer et al., 2010). The subsequent morphogenetic processes 76 that transform the otic vesicle into the intricate structure of the mature 77 ear are poorly understood, although differential proliferation and 78 apoptosis have been implicated (Lang et al., 2000). These morphogenetic 79 events must be tightly coordinated with cell fate acquisition to form a 80 functional ear and again, little is known about the molecular mechanisms 81 responsible.

Interestingly, Pax2 is prominently expressed in the ear and elsewhere 83 at sites where tissue outgrowth and shaping takes place (Dressler et al., 84 1990; Grote et al., 2006; Nornes et al., 1990; Rajakumar and Chamberlin, 85 2007), raising the possibility that Pax2 plays a role in regulating these 86 events. Here we test the hypothesis that Pax2 coordinates otic identity 87 and morphogenesis. We find that it is required for the expression of early 88 otic markers and that it independently controls epithelial integrity of 89 the placode. Downstream of Pax2, N-cadherin and N-CAM are required 90

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to maintain apical cell adhesion between otic cells as a prerequisite forplacode invagination.

### 93 Materials and methods

### 94 Embryo culture and electroporation

95Fertile hens' eggs were incubated in a humidified incubator at 96 38 °C until they had reached the appropriate stage (Hamburger and 97 Hamilton, 1951) (HH). Expression vectors and morpholinos were transfected into the head ectoderm using electroporation (McLarren 98 et al., 2003; Mende et al., 2008) and maintained in New culture (New, 99 1955; Stern and Ireland, 1981) for 16-27 h. Embryos were fixed in 4% 100 101 paraformaldehyde, 2 mM EGTA in phosphate buffered saline (PBS) overnight at 4 °C for in situ hybridisation or in 4% paraformaldehyde 102 in PBS for 30 min at room temperature for immunocytochemistry. 103

### 104 Morpholinos and expression constructs

All morpholinos (MO) were labelled with fluorescein conjugates 105(Gene Tools). MOs targeting Pax2 (Mende et al., 2008) and N-cadherin 106 (Shiau and Bronner-Fraser, 2009) were described previously. For 107 108 N-CAM two different MOs were designed targeting the translation start site (MO1: 5'-GCCGCTCCGAAATAGCCGTTCCGTG-3') and a splice 109 110 blocking MO targeting the boundary of exon 7 and intron 7 (MO2: 5'-AACAGGCAAAAGCTCACCAAAGACT-3'). Each N-CAM MO was tested 111 separately for efficient knock down (n=7 for MO1, n=8 MO2); the 112 113 experiments shown in the results section used electroporation of both MOs simultaneously. As control we used sense MO or standard control 114 MO (Gene Tools). The coding sequences of mouse Pax2 (Dressler et al., 1151990) and chick Sox2 (Rex et al., 1997) were cloned into pCAB-IRES-GFP 116 117(McLarren et al., 2003) to generate Pax2 and Sox2 expression constructs. 118We confirmed that the Sox2 expression construct produces Sox2 protein by performing Sox2 antibody staining (R&D) after electropora-119 tion into the cranial ectoderm. 120

### 121 In situ hybridisation and immunocytochemistry

Whole mount in situ hybridisation was performed as previously 122described (Streit et al., 1998) using DIG-labelled anti-sense probes for 123Eya1 (Chest668D18), Gata3 (Sheng and Stern, 1999), Pax2 (Streit, 124 1252002), Sox2 and -3 (Rex et al., 1997). Antibody staining was performed on cryosections as previously described (Bailey et al., 2006) using 126 polyclonal antibodies against Pax2 (Zymed) and B-catenin (Abcam) and 127 monoclonal antibodies against  $\alpha$ -catenin (BD Biosciences), N-cadherin 128 (Sigma) and N-CAM (5e; Developmental Hybridoma Bank). Appropri-129130ate secondary antibodies were coupled to Alexa 488, Alexa 594 or Cy5 (Invitrogen). Alexa 488 phalloidin was used to label actin and nuclei 131 were visualised using DAPI. Sections were analysed using a Leica TCS 132SP5 confocal microscope. The elongation index (length/width ratio) was 133 determined in individual GFP or MO<sup>+</sup> otic placode cells by measuring 134135their maximum length and width. Mean values and standard deviation 136were determined and the Mann-Whitney Rank Sum test was used to determine statistical significance. 137

### 138 Identification of putative Pax2 binding sites on the otic N-cadherin enhancer

Genomic sequences for human, mouse and chick N-cadherin loci 139 were downloaded from the Ensembl genome browser. A VistaPlot 140 alignment of the N-cadherin loci was cross-referenced to the position 141 and sequence of the known chick En2-DP enhancer (Matsumata et al., 1422005). VistaPlot yielded a ClustalW alignment for a conserved non-143 coding sequence corresponding to the En2-DP enhancer, from which a 144 consensus enhancer sequence was derived. Putative conserved 145transcription factor binding sites were identified within the consensus 146 147 enhancer sequence according to the consensus recognition sequences for Gata [A/T]GATA[A/G], Pax2 TNGTCA[C/T]GC[A/G]TGA and SoxB1, 148 ATTGTG. Of the putative binding sites identified, only those with the 149 highest cross-species conservation were annotated, as follows: Gata 150 >80% identity, Pax2 >50% identity and SoxB1 >65% identity. These 151 identity thresholds were chosen to reflect the length and heterogeneity of the individual consensus recognition sequences. 153

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### Results

### Pax2 is required for the specification of otic precursors

To establish whether Pax2 plays a role in the acquisition of otic fate in 156 amniotes we designed a knock down approach in chick using two 157 different morpholinos (MO). Otic identity was assessed by analysing the 158 expression of the earliest pan-otic markers Gata3, Eya1 and Sox2. Both 159 MOs, one targeting the translation start site and the other targeting a 160 splice junction, remove Pax2 protein effectively (Mende et al., 2008). 161 When electroporated into the otic territory at HH6-8<sup>-</sup> control MOs have 162 no effect (Fig. 1A, A', C, C', E, E'), while both Pax2 MOs produce identical 163 phenotypes: the expression of the otic placode markers *Gata*3 (15/22), 164 Eya1 (6/10) and Pax2 (22/22) is abolished (Fig. 1B, B', D, D', F, F'), but Sox2 165 expression is normal (n = 11; Fig. 1H, H'). To confirm that this effect is 166 specific, we coexpressed Pax2 with the splice blocking MO and find that 167 this rescues the loss of Gata3 expression (5/5; Fig. 1G, G'). Thus, in chick 168 Pax2 is necessary for the expression of some early otic-specific genes and 169 thus may play a role in conferring otic identity to cranial ectoderm. 170

### Pax2 induces Gata3, but not other early otic markers

In the eye, the Pax family member Pax6 acts as a 'master regulator' 172 inducing ectopic eyes when misexpressed (Gehring, 1996). To assess 173 whether Pax2 has similar properties in the ear we misexpressed Pax2 174 at HH6/7. Gata3 becomes dramatically upregulated in both the cranial 175 (18/33; Fig. 2D, D', d, d') and trunk (not shown) ectoderm, while Eya1 176 (n=9; Fig. 2B, B', b), Sox2 (n=8, not shown), Sox3 (n=7; not 177)shown) and Pax2 (n = 8; Fig. 2E, e') are unaffected. We never observe 178 ectopic placode-like structures or vesicles (see below) as seen with 179 other transcription factors like Spalt4 (Barembaum and Bronner- 180 Fraser, 2007). Surprisingly, Pax2 overexpression in the placode itself 181 abolishes the expression of Eval and Pax2, but not of Gata3 (Figs. 2B', 182 b', D', d', E, e). It is possible that the amount of Pax2 protein is critical 183 for normal gene expression as suggested by the dose dependent 184 function of Pax proteins in humans and mouse (for review see: Eccles 185 et al., 2002). Alternatively, overexpression of Pax2 may sequester 186 essential Pax2 co-factors and as a result downstream target gene 187 expression is lost. Together, these observations suggest that Pax2 is 188 not sufficient to impart otic character or placode morphology to non- 189 otic ectoderm. 190

Cell elongation and assembly of adherens junctions in the otic placode 191

Soon after otic-specific genes start to be expressed, otic precursor 192 cells noticeably change their shape, as seen in many other morphoge-193 netic events (Colas and Schoenwolf, 2001; Pilot and Lecuit, 2005; 194 Schoenwolf and Franks, 1984; Wallingford, 2005). To visualise these 195 changes in single cells, we electroporated GFP into the pre-otic domain 196 to generate mosaic expression and analysed cell shapes by confocal 197 microscopy. First, otic cells lengthen dramatically along their apical-198 basal axis, reflected by an increase in their elongation index (EI; 199 length/width ratio) from  $2.27 \pm 0.67$  (n = 8) and  $2.36 \pm 0.5$  (n = 5) at 200 the 5- and 6-somite stage respectively, to  $4.03 \pm 0.88$  (p = 0.0007; 201 n = 8) only 90 min later, at the 7 somite stage (Figs. 3A, B). Shortly 202 thereafter (HH10), we observe apical accumulation of cortical actin 203 (Fig. 3D; see also (Sai and Ladher, 2008) and around the 13 somite 204 stage, the key components of adherens junctions N-cadherin and  $\alpha$ - and 205  $\beta$ -catenin (Knust and Bossinger, 2002; Niessen and Gottardi, 2008; 206

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**Fig. 1.** Pax2 is required for otic marker expression.Control (A, A', C, C', E, E') or Pax2 MOs (B, B', D, D', F, F', H H') were electroporated into otic precursors at the 1–2 somite stage. At HH10–12, *Eya1* (A–B', a–b') and *Gata3* (C–D', c–d') expression is present in cells carrying control MOs (green in A, a, C, c; arrow heads), but absent in Pax2 MOs electroporated cells (green in B, b, D, d; arrow heads). Note in d': non-invaginated placode on targeted side. Pax2 protein expression is not affected by control MOs (E, E', e, e'; arrow head), but absent in cells with Pax2 MOs (F, F', f, f'; arrow head). Note the difference in cell shape of the control and Pax2 MO cells in e (white arrow head) and f (open arrow head). Loss of Pax2 does not affect *Sox2* expression in the otic placode (H, H', h'). *Gata3* expression is rescued when otic cells are co-electroporated with splice blocking MOs and a Pax2 expression construct (G, G', g, g'). Lines in A'–D', E, F, G' and H' indicate the level of sections shown in a–g, a'–g'.

Nishimura and Takeichi, 2009) assemble apically (Fig. 3D), while the cell
 adhesion molecule N-CAM accumulates just basal to the actin belt
 (Fig. 3C). Thus, cell elongation is followed rapidly by assembly of
 apical components necessary for apical constriction and subsequent
 invagination.

#### Pax2, N-CAM and N-cadherin are essential for placode integrity 212

In addition to changes in gene expression after gain or loss of Pax2 213 expression, we also observe changes in otic placode cell morphology 214 (compare Figs. 1e and f; 2e). We therefore characterised shape 215



**Fig. 2.** Pax2 is not sufficient to confer otic identify to ectodermal cells.Pax2-GFP (B, B', D, D', green; E, brown) or GFP (A, A', C, C', green) was misexpressed at the 0–1 somite stage. While *Gata3* expression is induced ectopically (D', d, d', arrow head), *Eya1* (B', b') and *Pax2* (E, e') are not. Pax2 misexpression in the otic placode leads to loss of *Eya1* (B', b; arrow head) and *Pax2* (E, e, arrow head), but expression of *Gata3* does not change (d). No effect is observed in control electroporated embryos (A', a, a', C', c). Black lines in A'–E indicate the level of sections shown in a–e and a'–e'.

**(** )



**Fig. 3.** Cell shape changes during otic placode formation and AJC assembly. A. GPF was electroporated into otic precursors to visualise individual cells. Otic cells elongate dramatically after the 6 somite stage and continue to do so over the next hours. Bottom row: five representative cells from each stage to illustrate elongation. B. The elongation index (length/width ratio; EI) changes significantly between 6 and 7 somites (\*\*\*p = 0.0007); mean values  $\pm$  standard deviation are shown. C. N-CAM is absent in otic precursors at 5 somites (open arrow); it is first observed at the 9-somite stage (arrow) and intensifies thereafter. At 16 somites, double staining with phalloidin shows N-CAM (open arrow heads) localisation just basal to apical actin demarcated by the white line in bottom right panel. D. The components of adherens junctions cadherin,  $\alpha$ -catenin and  $\beta$ -catenin assemble apically after the 10 somite stage.

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Fig. 4. Pax2 controls cell shape in the otic placode.A. Otic precursors were electroporated with control MOs or GFP (top), Pax2 MOs (middle) or Pax2-GFP (bottom). While control cells have elongated at the 13–15 somite stage, Pax2 loss and Pax2 overexpression (Pax2 OE) result in loss of placode cell morphology. Right: five representative cells for each condition. B. Compared to control electroporated cells (WT 13–15ss) the El is significantly reduced (\*\*\*) in cells electroporated with Pax2 MOs or Pax2-GFP (Pax2 OE). For comparison measurements from 6 somite placodes are included (WT 6ss). Graph shows mean values ± standard deviation.



**Fig. 5.** Pax2 is required for N-cadherin and N-CAM expression.A. Control electroporated otic placodes (top row) express N-cadherin (magenta) at the 15–16 somite stage. Loss of Pax2 (Pax2 MO, middle row) leads to loss of N-cadherin (open arrow heads, magenta), while N-cadherin is upregulated (white arrow heads) when Pax2 is misexpressed (Pax2 OE, bottom row). Note: N-cadherin is localised apically. B. Control electroporated otic placodes express N-CAM at the 12–13 somite stage (top row, magenta). Loss of Pax2 (Pax2 MO, middle row) leads to loss of N-CAM (open arrow heads, magenta). In contrast overexpression (Pax2 OE, bottom row) results in increased N-CAM (white arrow heads). Note: N-CAM is localised along the entire cell surface. C. N-CAM is not expressed in trunk ectoderm (control), however ectopic expression of Pax2 (Pax2 OE) in this tissue leads to upregulation of N-CAM in electroporated cells (green, white arrow heads), but not in non-electroporated neighbours (open arrow heads); \*indicates somite.

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changes in individual cells by electroporating Pax2 MOs into the pre-otic 216 217 domain of HH6/7 embryos. Unlike their wild-type neighbours, targeted cells fail to maintain their columnar shape: their EI is significantly 218 219reduced when compared to cells carrying control MOs (Figs. 4A, B) and they resemble the ectoderm before placode formation (Pax2 MO: 220EI  $2.03 \pm 0.78$ , n = 9; control MO: EI  $5.96 \pm 1.21$ , n = 11; p = 0.0001; 221 Fig. 4B). Since components of adherens junctions are required to 222maintain cell polarity and epithelial integrity (Nandadasa et al., 2009; 223224 Tinkle et al., 2008; Yang et al., 2009) we asked whether the lack of 225 columnar morphology is accompanied by loss of N-cadherin and N-CAM. Indeed, both cell adhesion molecules are absent when Pax2 is knocked 226down (N-cadherin: n = 7; N-CAM: n = 7; Figs. 5A–C). 227

To assess whether loss of N-CAM or N-cadherin can phenocopy the loss of Pax2 and whether these molecules themselves are critical for placode integrity, we used MOs to knock down their expression in the ear. Otic cells carrying N-cadherin or N-CAM, but not control MOs, 231 lose contact with their neighbours and fail to maintain an elongated, 232 columnar shape (Fig. 6; control MO: n = 16; N-CAM MO: n = 15; N- 233 cadherin MO: n = 10). At the 13–16 somite stage, their El is significantly 234 reduced compared to cells carrying control MOs (control: El 5.7  $\pm$  1.6; 235 N-cadherin: El 3.2  $\pm$  1.2; N-CAM: El 3.2  $\pm$  1.3; Figs. 6C and D). Together, 236 these results suggest that Pax2 controls integrity of the otic placode by 237 regulating apical cell adhesion via N-CAM and N-cadherin and that 238 both cell adhesion molecules are required independently to maintain 239 cell elongation. 240

Pax2 may control cell shape independent of otic fate

The above results show that Pax2 is required for expression of both  $_{\ 242}$  otic markers and cell adhesion molecules, but is unable to induce ectopic  $_{\ 243}$ 



**Fig. 6.** N-cadherin and N-CAM are required to maintain otic placode morphology. A. Loss of N-cadherin mimics the absence of Pax2. At the 16–18 somite stage, cells carrying control MOs show elongated shape (top row) and N-cadherin expression apically (magenta); panel on the right shows an overview of the same section with both placodes. In contrast, the placode does not thicken or invaginate in N-cadherin knock downs (bottom row, open arrow heads) and cells remain cuboidal. N-cadherin expression is lost (magenta) in the targeted side, but present on the contralateral side (panel on the right). B. Loss of N-CAM mimics the absence of Pax2. Control electroporated cells (top row) are elongated at the 13–15 somite stage and express N-CAM (red) and Pax2 (blue). In contrast, cells carrying N-CAM MOs (bottom row) are round (open arrow head), have lost N-CAM expression (red), but continue to express Pax2 (blue). Panels on the right show a low magnification to include the contralateral placode for comparison. C. Twenty representative cells from control, N-cadherin and N-CAM MO carrying cells show the difference in cell shape. G. The elongation index of cells expressing N-cAdherin or N-CAM MOs is significantly reduced when compared to control electroporated cells (WT 13–16ss). For comparison the El for placode cells from 6 somite embryos are included. Graph shows mean values ± standard deviation.

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otic fate. Is the acquisition of otic identity connected directly to the 244 245 control of adhesive properties or cell shape? When Pax2 is expressed ectopically, both N-cadherin (n=6) and N-CAM (n=10) are strongly 246 247induced compared to control electroporated cells, even in trunk ectoderm (Figs. 5A, C). However, we do not observe cell elongation. 248This is probably due to aberrant subcellular localisation of N-CAM: while 249N-cadherin is restricted apically as in the normal otic placode, N-CAM 250fails to do so but is spread along the entire cell surface (Figs. 5B, C). When 251252overexpressed in the otic placode itself, Pax2 also induces changes of cell 253morphology similar to those observed in the absence of Pax2 (Figs. 4A, B): their EI is reduced from  $5.96 \pm 1.21$  in controls (see above) to  $2.98 \pm$ 2541.06 (n = 15; p = 0.0001). Both N-cadherin and N-CAM (Figs. 5A, B) are 255upregulated, while otic markers are lost (see above Fig. 2). In addition, 256subcellular localisation of N-CAM is disturbed and placode organisation 257is disrupted (Fig. 5B). Thus, although Pax2 does not seem to be sufficient 258to initiate the otic programme in cranial ectoderm, it induces ectopic 259expression of N-cadherin and N-CAM in both locations. These findings 260 suggest that Pax2 may control cell shape and otic identity through 261independent mechanisms. 262

#### 263 Sox2 is not sufficient to rescue otic cell shape in the absence of Pax2

An otic-specific enhancer for N-cadherin has recently been charac-264 terised, whose activity depends on SoxB1 group binding sites 265266 (Matsumata et al., 2005; see supplementary Fig. 1). In addition, we have identified two evolutionary conserved, putative Pax2 binding sites 267in this enhancer, one very close to a SoxB1 group binding site (Fig. S1). 268To assess if the control of N-cadherin by Pax2 is mediated by Sox 269proteins we electroporated otic precursors with Pax2 MOs together 270271with full length Sox2. Sox2 is unable to rescue the Pax2 MO phenotype: 272the ectoderm remains cuboidal and N-cadherin is not expressed (Fig. 7). 273These findings show that Sox2 alone cannot restore N-cadherin expression and placode morphology in the absence of Pax2 function, 274suggesting that the factors may synergise to activate N-cadherin. 275

#### 276 Discussion

Pax2 is among the earliest genes to be expressed in the pre-otic 277field (Groves and Bronner-Fraser, 2000; Hans et al., 2004; Hidalgo-278Sanchez et al., 2000; Streit, 2002; Torres et al., 1996). Here we show 279280that in chick Pax2 plays a dual function as a key regulator of otic cell identity and shape. Pax2 function is required for the expression of 281 otic transcription factors and for cell adhesion molecules, which 282 in turn are necessary for epithelial integrity and subsequent placode 283 284invagination.

#### Pax2 and otic precursor specification

Commitment of ectodermal cells to an otic fate is reflected by the 286 sequential expression of transcription factors. Members of the Dlx and 287 Foxi1 families initially demarcate the pre-otic field. In zebrafish they 288 confer competence to respond to the otic inducing signal FGF (Hans 289 et al., 2007; Nissen et al., 2003; Solomon and Fritz, 2002; Solomon 290 et al., 2003, 2004). In response to FGFs, otic progenitors begin to 291 express Pax2 and Pax8 (Hans et al., 2004; Martin and Groves, 2006; 292 Wright and Mansour, 2003), which appear to cooperate in promoting 293 otic development (Hans et al., 2004). In amniotes, however, a role for 294 Pax proteins in otic specification has not yet been demonstrated. Pax2 295 mutant mice form an otic vesicle, but develop cochlear defects later 296 (Torres et al., 1996; Burton, 2004 #2421), while otic development is 297 Q1 merely delayed in Pax8 mutants (Christ et al., 2004); double mutants 298 have not been examined. Since both Pax genes encode highly related 299 transcription factors with common biochemical properties (Bouchard 300 et al., 2000; Pfeffer et al., 1998), the lack of an early ear phenotype in 301 either mutant is probably due to functional redundancy. Our finding 302 that loss of chick Pax2 alone leads to the absence of early otic markers 303 and of the placode itself seems to contradict the above results. 304 However, it is possible that Pax2 is the only Pax gene expressed early 305 during otic specification in birds. 306

The chromosomal region containing the Pax8 locus has undergone 307 considerable chromosomal rearrangement during evolution (Fan 308 et al., 2002a,b; Yunis and Prakash, 1982). In humans, the Pax8 locus 309 is found on 2q13–2q14.1, a region that arose through fusion of two 310 ancestral chromosomes (Yunis and Prakash, 1982) and analysis of the 311 syntenic regions in mammals, amphibians and fish reveals frequent 312 chromosomal rearrangements (AS, unpublished observations). While 313 in amphibians, medaka and stickleback the Pax8-containing region 314 clearly corresponds to that in mammals, the zebrafish Pax8 locus on 315 chromosome 5 shows no synteny with this region. In birds and 316 reptiles, however, the entire region is missing. It is therefore possible 317 that the Pax8 locus was lost in Sauropsids, providing an explanation 318 for why loss of Pax2 alone is sufficient to cause the loss of otic identity 319 in chick. We therefore propose that in birds Pax2 is the key Pax 320 protein controlling the specification of otic progenitor cells. 321

The Pax family member Pax6 plays a central role in eye formation 322 and is able to induce ectopic eyes in many species across the animal 323 kingdom (Gehring, 1996). Do other Pax proteins have similar 324 functions as master regulators of sensory placode formation? *Pax3* is 325 specifically expressed in the ophthalmic portion of the trigeminal 326 placode (Stark et al., 1997). While it is required for the specification of 327 trigeminal neurons, Pax3 is unable to induce them ectopically (Dude 328 et al., 2009). Our results suggest that Pax2 alone may not be sufficient 329



**Fig. 7.** Sox2 is not sufficient to rescue cell shape or placode invagination in the absence of Pax2.Otic precursors were electroporated with Pax2 MOs and Sox2 at HH6/7. Cell shape and invagination of the otic placode remain disturbed: compare the non-electroporated control side (left) and the Pax2 MO/Sox2 expressing contralateral side (green, open arrow head or arrow). Pax2 expression (top row, magenta, open arrow head) is absent in electroporated cells; Sox2 does not rescue N-cadherin expression (bottom row, magenta, open arrow). Panels on the right show higher magnification of the targeted area.

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to impart otic character to non-otic ectoderm: *Gata3*, but none of the
other otic markers tested, is upregulated in response to ectopic Pax2
expression. Thus, the ability to induce ectopic sensory structures
appears to be unique to Pax6.

#### 334 The Sox, Pax and Gata cassette as coordinator of fate and morphogenesis?

Our results uncover a novel role for Pax2 in controlling placode 335 336 morphology. When Pax2 expression levels are disturbed, otic cells fail to adopt columnar shape and instead remain cuboidal. The expression 337 338 of two apically localised cell adhesion molecules, N-cadherin and N-CAM, is disrupted and as a consequence the placode epithelium loses 339 340integrity and fails to invaginate. Consistent with the idea that Pax2 341 regulates cell morphology and invagination,  $\alpha$ -catenin,  $\alpha$ -actinin and several microtubule associated proteins have been predicted as 342 potential direct targets of Pax2 based on bioinformatic analysis 343 (Ramialison et al., 2008). Within the Pax family, both Pax6 and Pax3 344 have been implicated in controlling cell adhesion, morphology and 345 behaviour in the eye, neural crest cells and muscle (Buckingham and 346 Relaix, 2007; Collinson et al., 2000; Edelman and Jones, 1995; Holst 347 et al., 1997; Kallunki et al., 1995; Mayanil et al., 2000; Smith et al., 348 2009; Wiggan and Hamel, 2002). We therefore suggest that Pax 349 350 proteins play a fundamental role in development by integrating cell 351 fate allocation and morphogenetic events.

352It is likely however that Pax proteins cooperate with other transcription factors to control placode morphogenesis. After Pax2, 353 members of the SoxB1 family and Gata3 become expressed in otic 354355 progenitors. Concomitantly, cells elongate to acquire columnar shape and then invaginate into an otic cup. In mouse, Sox9 and Gata3 are 356 necessary for placode invagination (Barrionuevo et al., 2008; Lillevali 357 et al., 2006) and we suggest that these factors, together with Pax2 and 358 359 Sox2, control the expression of N-cadherin and N-CAM to maintain cell shape. The otic N-cadherin enhancer contains putative binding sites for 360 361 all three factors (Fig. S1 and Matsumata et al., 2005) and they may 362 therefore cooperate to initiate N-cadherin. Although the regulatory elements that control N-CAM expression in the ear have not been 363 identified, other N-CAM enhancers contain Pax binding sites (Edelman 364 365 and Jones, 1995; Holst et al., 1997).

Pax, Gata and SoxB1 group transcription factors are frequently 366 coexpressed at sites where cell fate acquisition and morphogenesis are 367 tightly controlled (Barrionuevo et al., 2008; Grote et al., 2006; Lillevali 368 et al., 2006; Matsumata et al., 2005; Rajakumar and Chamberlin, 2007; 369 Smith et al., 2009). SoxB1 and Pax proteins often synergise to control gene 370 expression. In the lens, Sox2 and Pax6 control  $\delta$ -crystallin and N-371 cadherin (Matsumata et al., 2005; Smith et al., 2009) and are 372 coexpressed with Gata3 (see Figs. 1 and 2; Sheng and Stern, 1999). 373 374Likewise, they control the activity of the diencephalic enhancer (N3) of Sox2 (Inoue et al., 2007), while Sox9 and -10 synergise with Pax3 to 375 activate neural crest and glia expression of Sox10 (Werner et al., 2007). 376 Although the role of Gata proteins in this context is less well established, 377 these factors are essential for endoderm invagination in C. elegans 378 379 (Sawyer et al., 2010). Thus, Pax, SoxB1 and Gata factors may emerge as 380 key coordinators of cell behaviour and fate.

#### 381 Cell adhesion molecules in otic cell morphology and invagination

382 Our studies reveal an essential role for two cell adhesion molecules, N-CAM and N-cadherin, in the maintenance of epithelial integrity and 383 invagination of the otic placode. While N-CAM plays important roles in 384 385 the developing and adult nervous system being involved e.g. in neurite outgrowth, synaptic plasticity and regeneration (Ditlevsen et al., 2008; 386 Edelman, 1985; Maness and Schachner, 2007), little is known about its 387 potential role in epithelial morphogenesis. N-CAM is best known for its 388 function as homophilic cell adhesion molecule in neuronal cells, but 389 recent evidence suggests that it also acts as a multifunctional regulator 390 391 of cell behaviour (Ditlevsen et al., 2008; Hansen et al., 2008) and

references therein). It regulates cytoskeletal dynamics by associating 392 with proteins like spectrin,  $\alpha$ - and  $\beta$  tubulin and  $\alpha$ -actinin and by 393 coupling membrane associated complexes to the cytoskeleton. It is 394 tempting to speculate that interactions similar to those that regulate 395 neurite outgrowth also modulate epithelial cell behaviour.

Cadherin-based adherens junctions are crucial for remodelling and 397 folding of epithelial sheets, for maintaining cell polarity and for tissue 398 integrity (D'Souza-Schorey, 2005; Gumbiner, 2005; Nishimura and 399 Takeichi, 2009). Transmembrane cadherins attach to cortical actin 400 through  $\alpha$ -,  $\beta$ - and  $\gamma$ -catenin (Hirano et al., 1987; Matsuzaki et al., 1990; 401 Nagafuchi and Takeichi, 1988; Ozawa et al., 1989), but are also actively 402 involved in the assembly of cortical F-actin (Nandadasa et al., 2009). 403 Cadherins therefore drive morphogenetic tissue movements such as 404 apical constriction in the neural tube. Our results support the idea that 405 N-cadherin, downstream of Pax2, plays a similar role in placode 406 invagination. During neurite outgrowth, N-CAM and N-cadherin 407 interact with common and distinct intracellular partners and both 408 modulate FGF-receptor signalling (for review see: Hansen et al., 2008). 409 In the otic placode, FGF signalling is critical for apical actin accumula- 410 tion (Sai and Ladher, 2008), raising the possibility that N-CAM and N- 411 cadherin not only mediate cell-cell adhesion, but may also influence 412 the signalling pathways involved in invagination. 413

In conclusion, our studies suggest that during development 414 transcriptional regulators like Pax proteins play a critical role not only 415 in assigning cell fate, but also in controlling morphogenetic events. Pax 416 proteins together with Sox and Gata factors may therefore provide the 417 missing link between signalling pathways that induce cell identity 418 and shaping of complex organs. 419

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#### Appendix A. Supplementary data

Supplementary data associated with this article can be found, in 429 the online version, at doi:10.1016/j.ydbio.2010.07.007. 430

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