

# *Spalt4* mediates invagination and otic placode gene expression in cranial ectoderm

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Vertebrate placodes are regions of thickened head ectoderm that contribute to paired sensory organs and cranial ganglia. We demonstrate that the transcription factor *Spalt4* (also known as *Sall4*) is broadly expressed in chick preplacodal epiblast and later resolves to otic, lens and olfactory placodes. Ectopic expression of *Spalt4* by electroporation is sufficient to induce invagination of non-placodal head ectoderm and prevent neurogenic placodes from contributing to cranial ganglia. Conversely, loss of *Spalt4* function in the otic placode results in abnormal otic vesicle development. Intriguingly, *Spalt4* appears to initiate a placode program appropriate for the axial level but is not involved in later development of specific placode fates. Fgfs can regulate *Spalt4*, since implantation of Fgf2 beads into the area opaca induces its expression. The results suggest that *Spalt4* is involved in early stages of placode development, initiating cranial ectodermal invagination and region-specific gene regulatory networks.

**KEY WORDS:** *SALL4*, *Sox10*, Electroporation, Placode formation

## INTRODUCTION

In vertebrate embryos, ectodermal placodes are discrete regions of thickened epithelium that form transiently on the head ectoderm. They can be subdivided into two broad categories: sensory placodes that contribute to paired sense organs (nose, ear, lens, lateral line) and neurogenic placodes that contribute to cranial sensory ganglia (trigeminal, epibranchial) (Le Douarin et al., 1986; Webb and Noden, 1993). Initially, all placodes arise from a horse-shoe shaped ‘preplacodal domain’ at the border of the rostral neural plate prior to neurulation (Baker and Bronner-Fraser, 2001; Brugmann and Moody, 2005; Streit, 2004). They subsequently segregate into separate domains designated, from rostral to caudal, as olfactory, lens, trigeminal, otic and epibranchial, each with a distinctive gene expression profile. For example, all placodes express characteristic Pax genes, with the most rostral (nose, lens) expressing *Pax6*, intermediate level (trigeminal) expressing *Pax3* and most caudal (ear, epibranchial) expressing *Pax2* (Baker and Bronner-Fraser, 2001). After thickening of the ectoderm, placodal cells either invaginate or ingress to internalize as the first step in their conversion from epithelia to sensory structures.

Although a number of genes have been implicated in specification of placodal identity, little is known about what imbues placodal ectoderm with the ability to internalize, migrate and contribute to sensory organs and/or ganglia and thus distinguishes it from non-placodal ectoderm. At early stages, ectoderm from other axial levels is competent to form particular placodes when heterotopically grafted in place of the endogenous placode. However, this broad potential to respond to placode-inducing signals becomes limited with time (Groves and Bronner-Fraser, 2000). One intriguing possibility is that a factor(s), initially expressed throughout the cranial ectoderm is critical for invagination of ectodermal cells in response to placode inducing signals and that this becomes restricted with time to individual placodes.

Here, we show that a zinc finger transcription factor of the spalt gene family (Sweetman and Munsterberg, 2006), chick *Spalt4* (also known as *Sall4*), fulfills these criteria in the cranial ectoderm. Although its early expression is uniform throughout the head ectoderm, its localization later becomes restricted to the otic, lens and olfactory placodes. In *Drosophila*, mutations in members of the spalt gene family cause defects in both migration and cell fate (de Celis et al., 1999; Elstob et al., 2001; Kuhnlein and Schuh, 1996; Rusten et al., 2001). In *Xenopus*, *spalt* genes have been shown to be involved in brain development (Onai et al., 2004) and limb regeneration (Neff et al., 2005). Here, we show that expression of *Spalt4* in non-placodal ectoderm is sufficient to induce invagination or ingression of cranial ectodermal cells. Knockdown of *Spalt4* function in the otic placode results in deficient otic vesicle development whereas overexpression causes ear abnormalities. These results suggest that *Spalt4* is important for otic vesicle formation, and may be generally important for the invagination/ingression of placodal ectoderm.

## MATERIALS AND METHODS

### Expression constructs

We cloned the complete coding region of *Spalt4* into pCIG, which drives expression through a chicken actin promoter and CMV enhancer (Megason and McMahon, 2002). It also contains GFP driven from an IRES sequence downstream of the coding sequence, allowing for coexpression of the desired protein and GFP in the same cell. The sequence coding the amino terminus of *Spalt4* including the CCHC zinc finger (amino acids 1–98) was cloned into pCS2+NLS in frame with the nuclear localization signal. The fragment with the NLS and truncated *Spalt4* was then cloned into pCIG. The *Sox10* expression construct was described previously (McKeown et al., 2005). Control electroporations used either empty pCIG or empty pMES (in the *Sox10* overexpression experiments) plasmids.

### Ectoderm electroporations

The plasmid was injected into embryos containing three to seven somites (stages 8–9), as well as at later stages (stages 12–13) where indicated. The concentration of DNA was between 2 and 2.5  $\mu\text{g}/\mu\text{l}$ . Eggs were windowed and inked using standard procedures. The needle was placed through the vitelline membrane at the caudal end of the embryo nearly parallel to the embryo, and moved rostrally between the vitelline membrane and the embryo until it lay above the midbrain. The DNA was then injected using a glass needle until the embryo was completely covered with the DNA

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solution, and then withdrawn. The electrodes were constructed in the lab and were made of two pieces of platinum wire (19 gauge) bent at 45° from the horizontal, about 5 mm from the end and 4 mm apart. The positive platinum electrode was inserted through a hole at the edge of the blastoderm and under the embryo. The negative electrode, 4 mm away from the positive electrode, was placed on top of the vitelline membrane above the embryo at the level of the hindbrain and submerged in albumin but without touching the membrane. Three pulses of 8-10 V for 30 mseconds duration were applied 100 mseconds apart. The electrodes were carefully removed and the egg was sealed and incubated at 38°C for up to 72 hours.

#### Bead implants

Stage 4 embryos were collected on Whatman filter paper rings and turned ventral side up in Ringer's solution. A small slit was made in the area opaca next to the area pellucida. A bead soaked in 50 µg/ml Fgf2 or BSA (bovine serum albumin) was inserted into the slit (Litsiou et al., 2005) and incubated in modified New culture (Chapman et al., 2001) for 5-7 hours and then collected and fixed overnight in 4% paraformaldehyde.

#### Analysis of embryos

Embryos were collected in Ringer's solution and fixed in 4% paraformaldehyde overnight. Embryos were washed in PBT and embedded in gelatin for histochemical analysis or dehydrated in methanol for in situ hybridization. In situ hybridization was performed as described previously (Wilkinson, 1992). In situ hybridization on sections was performed using embryos fixed in Carony's fixative, embedded in paraffin and sectioned at 10 µm. In situ hybridization was performed using published procedures (Etchevers et al., 2001).

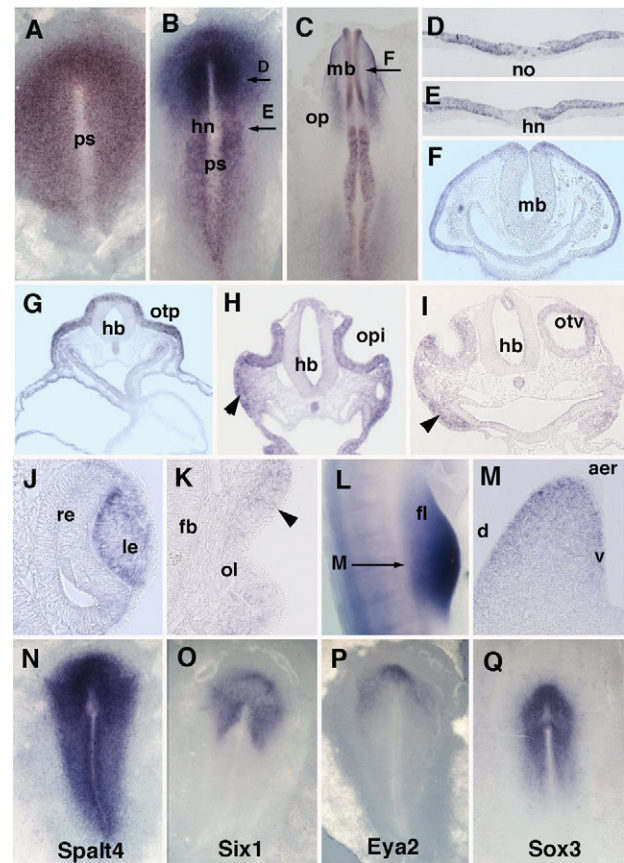
#### Antibodies

The anti-GFP (Abcam), anti-Pax2 (Zymed), anti-Pax6 (Covance), HuC/D (Molecular Probes) and TUJ1 (Covance) antibodies were obtained commercially. The *Spalt4* antibody was generated using a GST fusion protein construct that included the region encoding amino acids 654-835 of chicken *Spalt4*. This region lies between two zinc finger regions and has low sequence homology to other *spalt* genes. The antibody recognizes nuclei in tissues that express *Spalt4* RNA but does not recognize cells electroporated with *Spalt1*-expressing constructs. The pan-Dlx antibody was a kind gift from Jhumuku Kohtz, which was made from a construct from Grace Panganiban (Dong et al., 2000). The polyclonal antibody to Dlx3 (Bailey et al., 2006) gives specific nuclear staining in tissues that normally express *Dlx3*, such as the otic vesicle and the olfactory epithelium. However, it does not stain other CNS structures that express other *Dlx* genes (e.g. *Dlx1*, 2, 5 and 6). Primary antibodies were visualized with Alexa Fluor 488-conjugated donkey anti-goat or Alexa Fluor 594-conjugated donkey anti-rabbit secondary antibodies (Molecular Probes). TUNEL analysis was done using the In Situ Cell Death Detector, TMR kit from Roche according to the manufacturer's instructions.

## RESULTS

### *Spalt4* is expressed in cranial ectodermal placodes

We examined the pattern of *Spalt4* expression by in situ hybridization of chicken embryos from the early primitive streak stage (stage 3) to the time of formation of the otic and nasal placodes (E2 and E3). At stage 3, *Spalt4* is expressed in the epiblast throughout the embryo but absent from the hypoblast (Fig. 1A). As the node begins to regress (stage 5), there is increased expression in the presumptive neural plate above the notochord and some scattered cells within the notochord as well as continued expression in the ectoderm (Fig. 1B,D,E). *Spalt4* expression in stage 5 embryos (Fig. 1A,N) overlaps the expression of *Six1* (Fig. 1O) and *Eya2* (Fig. 1P) in the preplacode ectoderm and *Sox3* (Fig. 1Q) in the prospective neural plate. Later, at the open neural plate stage (stage 8), *Spalt4* expression is found throughout the head ectoderm and the open neural plate but is lost from the closing neural tube and non-head ectoderm (Fig. 1C,F). The otic placode strongly expresses *Spalt4* at

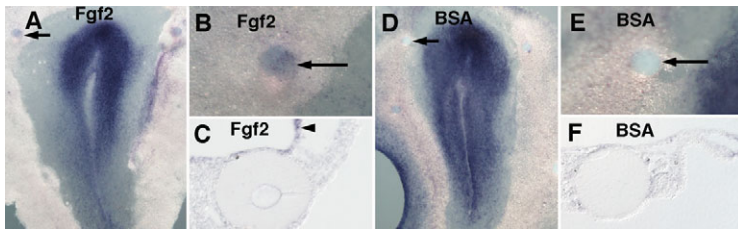


**Fig. 1. Expression of *Spalt4* in ectoderm and derived tissues.**

Whole-mount in situ hybridization of (A) stage 3 chicken embryo, (B) stage 5 chicken embryo (arrows indicate level of sections in D and E), (C) stage 8 chicken embryo (arrow indicates levels of sections in F). (D) Section through embryo in (B) at the level of the notochord. (E) Section through embryo in B at the level of Hensen's node. (F) Section through stage 8 embryo at midbrain level. (G) Expression of *Spalt4* in a stage 10 embryo sectioned at hindbrain level. (H) Expression of *Spalt4* in the otic pit (opi) of a stage 13 embryo. Arrowhead points to the neural crest. (I) Expression of *Spalt4* in the otic vesicle (otv) of a stage 16 embryo. Arrowhead indicates the neural crest. (J) Section through a stage 14 embryo at eye level. (K) Section through a stage 17 embryo at forebrain level. (L) Whole-mount in situ hybridization with *Spalt4* in a stage 19 embryo showing strong *Spalt4* expression in the forelimb. Arrow shows the level of the section in M. (M) Section through the forelimb in L. (N-Q) Comparison of stage 5 expression of (N) *Spalt4* (O) *Six1* (P) *Eya2* and (Q) *Sox3*. ps, primitive streak; hn, Hensen's node; no, notochord; otp, otic placode; opi, otic pit; otv, otic vesicle; mb, midbrain; hb, hindbrain; re, retina; le, lens; fb, forebrain; ol, olfactory epithelium; fl, forelimb; d, dorsal; v, ventral; aer, apical ectodermal ridge.

stage 10 (Fig. 1G) but has no detectable signal in the hindbrain. At stages 13 and 15 (Fig. 1H,I) *Spalt4* expression in the ectoderm is strong in the otic pits and continues as they close to form the otic vesicle, but becomes reduced in the otocyst by stage 18 (data not shown). At these stages, expression is observed in the neural crest (arrows in Fig. 1H,I) as previously described (Barenbaum and Bronner-Fraser, 2004). *Spalt4* can also be detected in the lens (Fig. 1J) and weakly in the olfactory epithelium (Fig. 1K). It is also present in limb mesoderm (Fig. 1L), but not the ectoderm or apical ectodermal ridge (Fig. 1M).



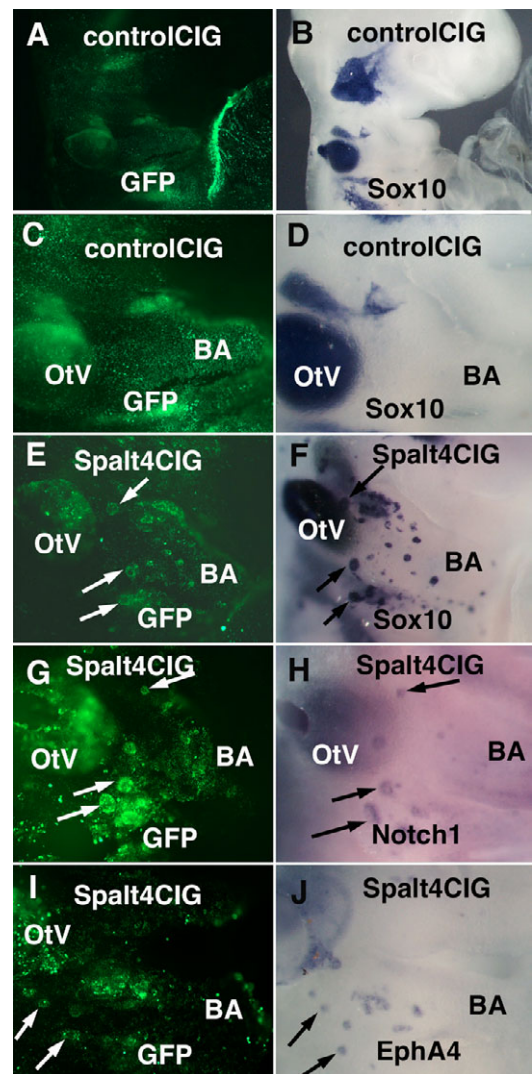


**Fig. 2. Fgf2-soaked bead induces *Spalt4*.** (A) Whole-mount in situ hybridization 5 hours after Fgf bead implantation. Arrow points to the bead. (B) Higher magnification of bead in (A). Arrow shows plane of section in C. (C) Section of region around bead in B. Arrowhead points to *Spalt4* signal. (D) Whole-mount in situ hybridization five hours after BSA bead implantation. Arrow points to bead. (E) Higher magnification of region around bead in D. Arrow shows plane of section in F. (F) Section of region around bead in E.

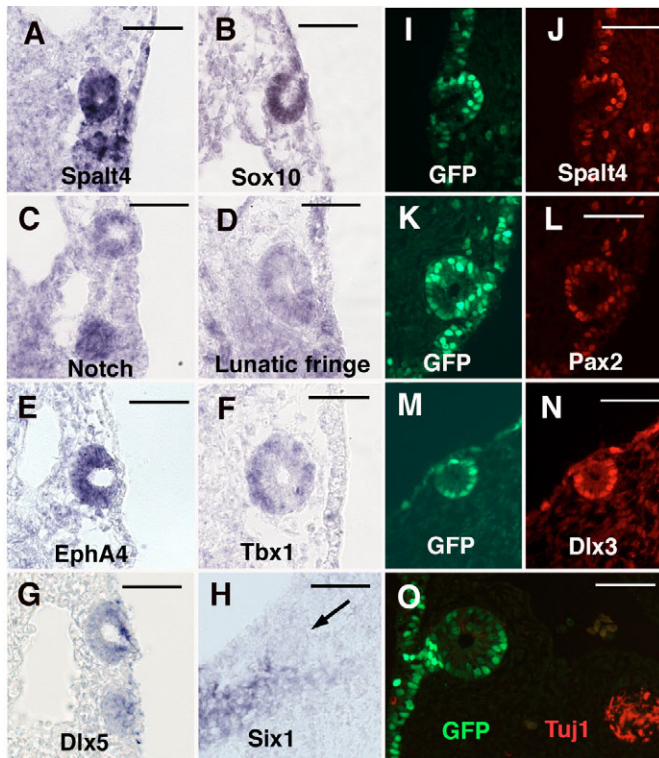
Fgf signaling has been implicated in placode formation (Litsiou et al., 2005; Maroon et al., 2002; Martin and Groves, 2006) as well as induction of the otic vesicle (Ladher et al., 2000; Ladher et al., 2005; Liu et al., 2003; Maroon et al., 2002; Vendrell et al., 2000). Furthermore, insertion of Fgf8-coated beads in the area opaca results in the induction of early placode markers such as *Dlx5*, *Sox3* and *Eya2* (Litsiou et al., 2005). To analyze the ability of Fgf signaling to induce *Spalt4*, we implanted an Fgf2-soaked bead (Litsiou et al., 2005) under the area opaca of stage 4 embryos, as this region of ectoderm is competent to respond to placode-inducing signals. Embryos were analyzed for *Spalt4* expression 5 to 7 hours thereafter. *Spalt4* was detected in the ectoderm surrounding the bead in nine out of ten embryos (Fig. 2A,B,C), while none was detected in control embryos (0/8 embryos) in which a BSA-soaked bead was implanted (Fig. 2D-F). In addition to *Spalt4*, the preplacodal marker *Eya2* was induced by Fgf2 (3/5) (data not shown).

### Misexpression of *Spalt4* leads to formation of ectopic vesicles in non-placodal ectoderm adjacent to the ear

To investigate its potential role in placode development, we used electroporation to misexpress a *Spalt4* construct that co-expressed GFP in portions of the cranial ectoderm that do not normally contribute to placodes. We always performed parallel electroporations using an empty pCIG vector as controls. As a first step, *Spalt4* was targeted to the ectoderm adjacent to the hindbrain of stage 8 chick embryos and up to as late as stage 13. One day after electroporation, we detected GFP-positive cells throughout the ectoderm (data not shown) including cells that will give rise to otic, trigeminal and epibranchial placodes as well as epidermis. As early as 30 hours we were able to detect ectopic vesicles (8/8). By 2 days after electroporation, we noted the formation of multiple small ectopic pits or vesicles outside the endogenous otic forming region (Fig. 3) in most *Spalt4*-electroporated embryos (123/127; Fig. 3E,G,I) and almost never in those electroporated with the control plasmid (1/49; Fig. 3A,C). The numbers and locations of these vesicles varied from embryo to embryo but 60% of *Spalt4*-electroporated embryos had five or more ectopic vesicles. The ectopic vesicles were found both rostral and caudal to the endogenous otic vesicle as well as laterally in the ectoderm overlying the branchial arches. Most of these vesicles were small, ranging from five to ten cells in diameter, although a few were as large as 30 cells in diameter. The ectoderm in the ectopic vesicles was generally monolayered, but often appeared thickened compared to the adjacent non-electroporated ectoderm. After 72 hours the ectopic vesicles were sometimes several cell layers thick (data not shown). All of the cells within the ectopic vesicles were GFP-positive. However, other GFP-positive cells remained in the adjacent ectoderm and failed to invaginate and also failed to express otic-specific genes (Fig. 3). Owing to the transient nature of electroporation, we were unable to detect GFP-expressing cells after 96 hours. Ectopic vesicles could be detected in embryos electroporated as late as stage 12 (4/4).



**Fig. 3. Electroporation of a plasmid driving *Spalt4* expression induces the expression of ectopic vesicles.** (A) Embryo electroporated with a control GFP plasmid. (B) Embryo in A hybridized with *Sox10* RNA probe. (C) Magnified view of embryo in A. (D) *Sox10* in situ hybridization of control embryo in C. (E) Embryo electroporated with *Spalt4*-GFP construct. A number of ectopic vesicles are visible (arrows). (F) The ectopic vesicles in the *Spalt4* electroporated embryo in E express *Sox10* (arrows). (G) Embryo electroporated with *Spalt4*-GFP construct. A number of ectopic vesicles are visible (arrows). (H) The ectopic vesicles in the *Spalt4* electroporated embryo in G express *Notch1* (arrows). (I) Embryo electroporated with *Spalt4*-GFP construct. A number of ectopic vesicles are visible (arrows). (J) The ectopic vesicles in the *Spalt4* electroporated embryo in (I) express *EphA4* (arrows). OtV, otic vesicle; BA, branchial arch.



**Fig. 4. Expression of otic expressed genes in the ectopic vesicles at the level of the hindbrain.** (A) *Spalt4*, (B) *Sox10*, (C) *Notch1*, (D) *Lunatic fringe*, (E) *EphA4*, (F) *Tbx1*, (G) *Dlx5*. (H) *Six1* mRNA was not detected in an ectopic vesicle by in situ hybridization (arrow), though surrounding cells were positive. (I) GFP. (J) An antibody to *Spalt4*. The same section as in I. (K) GFP. (L) Same section as in K showing *Pax2* expression. (M) GFP. (N) The same section as in M showing *Dlx3* expression. (O) An ectopic vesicle in an embryo 72 hours post electroporation, with the *Spalt4* expression construct expressing GFP (green) but there is little detectable TUJ1 staining (red). A portion of an adjacent ganglion shows strong TUJ1 staining. A-F show section in situ hybridizations. G and H are 20  $\mu\text{m}$  cryostat sections of whole-mount in situ hybridizations. The embryos in I-N were paraformaldehyde fixed 48 hours (I-N) or 72 hours (O) after electroporation then sectioned at 10  $\mu\text{m}$  on a cryostat and used for immunohistochemistry. Scale bars: 50  $\mu\text{m}$ .

### Ectopic vesicles at the level of the hindbrain express otic marker genes

We next tested whether the ectopic vesicles and pits resembled otic vesicles using a number of molecular markers for placodes in general [e.g. the *Six-Eya-Dach* gene network (Streit, 2004)], early otic markers such as *Pax2* (Groves and Bronner-Fraser, 2000), *Dlx3* (Pera and Kessel, 1999), *Nkx5.1* (Herbrand et al., 1998) as well as other transcription factors such as *Sox8* (Bell et al., 2000), *Sox10* (Cheng et al., 2000), *Dlx5* (Streit, 2002), *Tbx1* and *Tbx3* (Chapman et al., 1996) and signaling molecules such as *Bmp4*, *Notch1* and *Lunatic fringe* (Adam et al., 1998; Cole et al., 2000) thought to be involved in otic pit/vesicle or later ear development. By using a combination of immunocytochemistry and in situ hybridization, we examined the extent to which these ectopic vesicles mimicked the normal otic vesicle program of gene expression.

*Spalt4*-induced ectopic vesicles at the level of the hindbrain expressed *Sox10* (Fig. 3F), *Notch1* (Fig. 3H) and *EphA4* (Fig. 3J), though these markers were missing in electroporated ectoderm of control embryos (*Sox10* in Fig. 2D and data not shown for *Notch1*

**Table 1. Gene expression in the ectopic vesicles**

Gene	48 hours	30 hours	18 hours
<b>Preplacode</b>			
<i>Six1</i>	0/3		
<i>Six3</i>	0/2		
<i>Six4</i>	0/4		
<i>Eya1</i>	0/6		
<i>Eya2</i>	0/2		
<i>Eya4</i>	0/3		
<i>Dach1</i>	0/2		
<i>Dach2</i>	1/2		
<i>Dlx3</i>	3/3		
<i>Dlx5</i>	4/4		
<i>Irx1</i>	2/2		0/3
<i>Irx2</i>	2/2		
<b>Placode</b>			
<i>Sox10</i>	11/11	4/4	2/4
<i>Sox8</i>	3/3		
<i>Notch1</i>	8/8		
<i>EphA4</i>	6/6	4/4	3/3
<i>Lun. fr.</i>	6/6		
<i>Tbx1</i>	3/3		
<i>Tbx3</i>	2/2		
<i>Pax2</i>	5/5		
<i>Nkx5.1</i>	4/4		
<i>Bmp4</i>	5/5		
<i>Bmp7</i>	2/2		
<i>Gata2</i>	2/2		

Embryos were scored as positive if some of the ectopic vesicles, or some ectopic structure in the 18 hour post-electroporation, expressed the gene. *Tbx1* and *Tbx3* had signal only in the post-otic ectoderm. Genes were considered pre-placode if they were expressed early and believed to be important in placode formation. Genes were considered placode genes if they were expressed as the otic placode forms or later.

or *EphA4*). In situ hybridization performed on tissue sections showed that *Spalt4* was expressed throughout the ectopic vesicles (Fig. 4A). Similarly, *Sox10* (Fig. 4B), *Notch1* (Fig. 4C), *Lunatic fringe* (Fig. 4D), *EphA4* (Fig. 4E), *Tbx1* (Fig. 4F) and *Dlx5* (Fig. 4G) were expressed throughout the ectopic vesicles, though *Tbx1* was only detected in ectopic vesicles caudal to the otic vesicle. Interestingly, in the ectopic vesicles, with the exception of *Dach2*, we were unable to detect genes in the *Six-Eya-Dach* network (Table 1), including *Six1* (Fig. 4H).

In order to provide a marker independent of the IRES-GFP to verify which cells express the *Spalt4* transgene, we produced a polyclonal antibody to a unique region of chicken *Spalt4* protein (covering amino acid 654-835) allowing us to discriminate between expressing and non-expressing cells at high resolution. We found a good correlation between the expression patterns of *Spalt4* and GFP proteins (compare Fig. 4I,J). GFP in the ectopic vesicles also correlated with *Pax2* (Fig. 4K,L) and *Dlx3* (Fig. 4M,N) protein expression. By contrast, we were unable to detect the early neuronal marker, NeuroD, 48 hours following electroporation or neuron-specific  $\beta$ -tubulin (TUJ1) 72 post-electroporation (Fig. 4O). Cytokeratin 19 was downregulated in the ectopic vesicles, but not in other parts of the GFP-positive ectoderm, possibly because of indirect effects (data not shown).

### Overexpression of *Spalt4* in the otic placode causes severe ear abnormalities

The above results show that expression of *Spalt4* in naïve ectoderm causes ectopic vesicle formation. We next investigated whether excess *Spalt4* would alter development if overexpressed in the endogenous placode region. To this end, we targeted



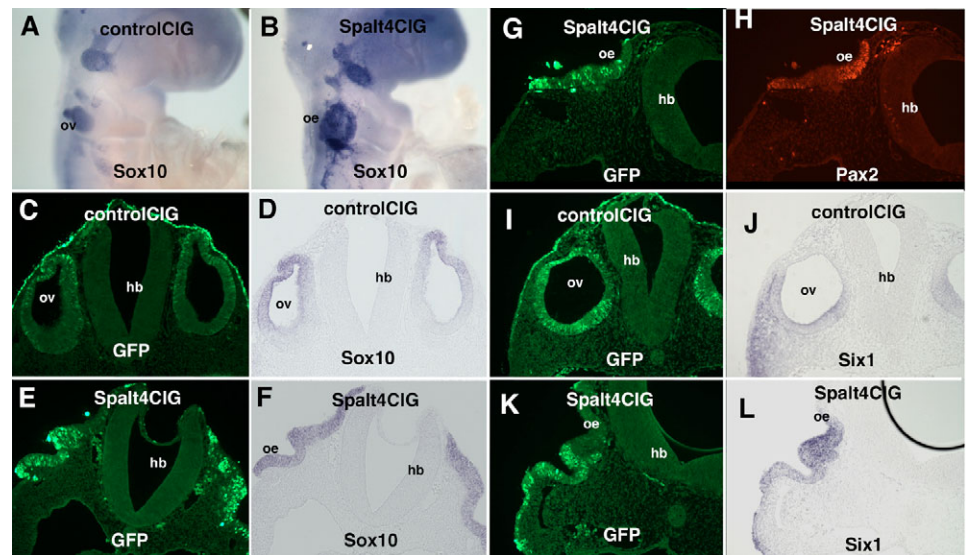
### Fig. 5. Misexpression of *Spalt4* in otic placodes can prevent the formation of otic vesicles.

(A) An embryo electroporated with a control plasmid gives rise to a normal otic vesicle (ov) expressing *Sox10*. (B) An embryo electroporated with a *Spalt4*-expressing plasmid gave rise to a flat ectoderm (oe) expressing *Sox10* where the otic vesicle is normally located.

(C) Section through an embryo electroporated with a control plasmid at the otic vesicle level shows GFP expression in the closed otic vesicle.

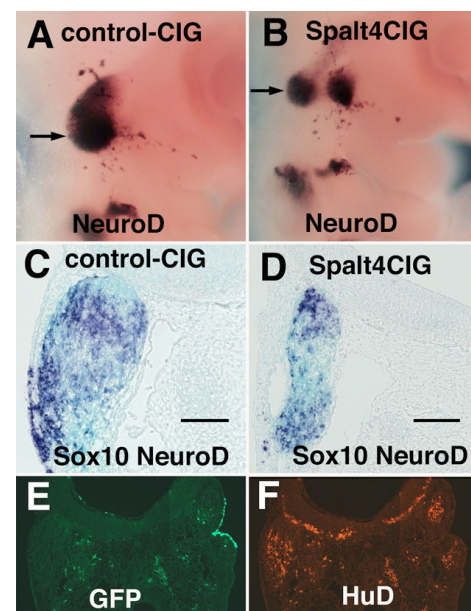
(D) Same section as in C showing expression of *Sox10* in the lateral half of the otic vesicle. (E) Section through an embryo electroporated with a *Spalt4*-expressing plasmid at otic vesicle level shows GFP-positive cells in the thickened epithelium where the otic vesicle would normally be located.

(F) In situ hybridization with *Sox10* RNA probe. Same section as in E. (G) Section through an embryo electroporated with a *Spalt4*-expressing plasmid at otic vesicle level shows GFP expression in the thickened epithelium where the otic vesicle would normally be located. (H) Same section as in G using a Pax2 antibody. (I) Section through otic vesicles of an embryo electroporated with a control plasmid. (J) Same section as in (I) showing *Six1* expression in the ventral half of the otic vesicle. (K) Section through the otic ectoderm of an embryo electroporated with a plasmid overexpressing *Spalt4*. (L) *Six1* expression in the same section as in (K). ov, otic vesicle; oe, otic ectoderm; hb, hindbrain.

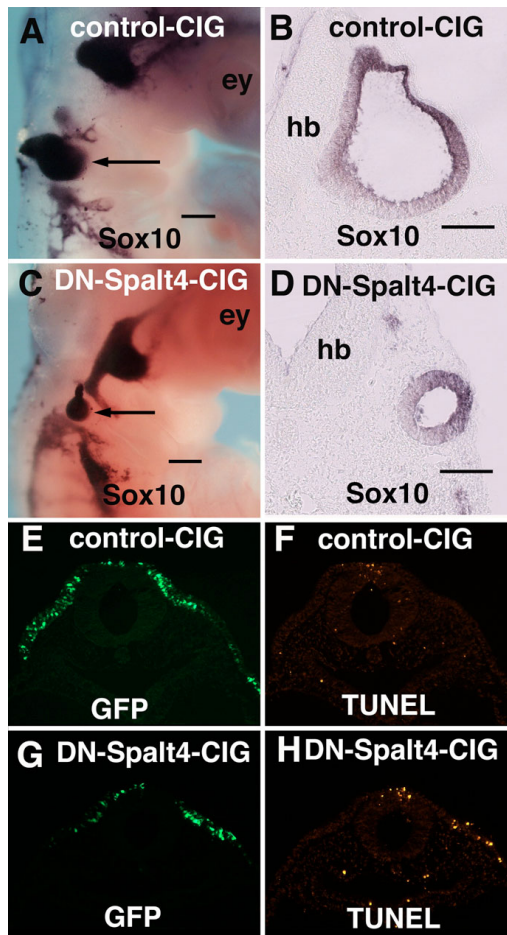


electroporations to the otic placode itself. This resulted in profound morphological malformations in the developing ear. The otic epithelium appeared enlarged and failed to close properly. Frequently, electroporation of *Spalt4* into the otic placode resulted in a flat or opened otic pit (29/42) rather than closed otic vesicles as in control embryos (0/31 with abnormal vesicles). The morphological alterations were variable, perhaps due to differences in amount or distribution of construct, but our data cumulatively suggest that excess *Spalt4* causes severe patterning defects in the developing ear. Molecular markers that selectively localize in different domains within the developing ear confirmed that cells overexpressing *Spalt4* assumed gene expression characteristic of the ear, though the spatial distribution was sometimes altered. After electroporation of *Spalt4*, *Sox10*, which is usually spatially restricted to the dorsal-lateral half of the otocyst (Fig. 5A,C,D), was expressed throughout the thickened ectoderm (Fig. 5B,E,F). *Sox10* expression was not limited to the cells expressing GFP (compare Fig. 5E with F), suggesting the effects may be non cell-autonomous, perhaps as a result of mispatterning of the otic ectoderm and failure to downregulate endogenous *Sox10* in the ventral-medial half as happens in control embryos. Similarly, *Pax2*, normally expressed medially in control electroporated otocysts, was observed in lateral regions after *Spalt4* overexpression (Fig. 5G,H). However the expression of *Six1*, which is not induced by *Spalt4* overexpression in the ectopic vesicles (Fig. 4H), remains expressed in a spatially restricted manner in the thickened otic ectoderm (Fig. 5K,L). In rare cases (<5%), multiple smaller vesicles were observed (data not shown). In contrast to electroporation at early stages, those performed at later times (stage 12 and 13) show normal otic vesicle formation (0/5 abnormal).

At the level of the midbrain, we observed some thickened ectoderm and a few ectopic vesicles after electroporation. In addition, *Spalt4* electroporated ectoderm failed to contribute to the trigeminal ganglia at the same levels as did stage-matched control electroporated trigeminal placode ectoderm. This resulted in the



**Fig. 6. The effect of expression of *Spalt4* in the trigeminal placode.** Whole-mount in situ hybridization with a probe to *NeuroD* in embryos electroporated with (A) control plasmid or (B) plasmid overexpressing *Spalt4*. Arrows point to the approximate plane of section of trigeminal ganglia in C and D. (C,D) Sections at trigeminal ganglion level of double in situ hybridizations with *Sox10* (light blue) and *NeuroD* (dark blue) in embryos electroporated with (C) control plasmid or (D) plasmid overexpressing *Spalt4*. (E,F) Sections through an embryo electroporated with *Spalt4* on the right side only, and analyzed with GFP (E), or HuD (F) antibody. Scale bars: 100  $\mu$ m.



**Fig. 7. Expression of a dominant-negative *Spalt4* constructs in the otic placode reduces the size of the otic vesicle.** (A) Whole mount and (B) section of an embryo electroporated with the control plasmid and hybridized in situ with a *Sox10* RNA probe. (C) Whole mount and (D) section of an embryo electroporated with the dominant-negative *Spalt4* plasmid and hybridized in situ with a *Sox10* RNA probe. (E) Embryo 8 hours post-electroporation with control plasmid and analyzed with GFP. (F) Same section as in E analyzed for cell death with TUNEL. (G) Embryo 8 hours post-electroporation with dominant-negative *Spalt4* plasmid and analyzed with GFP. (H) Same section as in G analyzed for cell death with TUNEL. Scale bars: 200 μm (A,C) 100 μm (B,D).

reduction of the placodal contribution to the trigeminal ganglia as evidenced by a reduction in *NeuroD* expression in whole mounts by in situ hybridization (Fig. 6A-D). This was particularly apparent in the ophthalmic branch, as well as in a split in the maxillomandibular branch (Fig. 6B,D,F). In addition, the *Spalt*-expressing cells at midbrain level failed to express either *Pax2* or *Pax6* proteins (data not shown). Further rostrally, GFP-positive cells maintained *Pax6* expression but failed to express *Prox1* or  $\delta$ -crystallin, which are characteristic of the lens (data not shown).

### Loss of *Spalt4* function in the otic placode leads to deficient vesicle formation

To examine the loss-of-function phenotype of chick *Spalt4*, we designed a truncated construct encoding the first zinc finger of *Spalt4* at the amino terminus. A similar truncated construct was previously used in *Xenopus* embryos to knock-down function of the

*spalt* protein XsalF and shown to function as a dominant negative (Onai et al., 2004). Constructs were introduced into the presumptive otic epithelium by electroporation in a similar manner to that used for the full-length construct.

The majority of embryos examined 2 days after electroporation with the dominant-negative construct (19/22) had significant reduction in the size of one or both otic vesicles after efficient levels of electroporation (as judged by high levels of GFP expression; Fig. 7C,D). By comparison, few embryos (4/25) electroporated with GFP-vector alone had smaller ears and none of these were as small as the experimental ears (Fig. 7A,B). GFP staining revealed uniform distribution of the electroporated construct throughout the ectoderm of both control and experimental embryos, including the miniature otic vesicles (data not shown). The reduced size of the vesicle in dominant-negative embryos was particularly dramatic in the rostrocaudal dimension, sometimes giving it a tightly squeezed look. There was a reduction of 30% ( $P < 0.0001$ ) in the length of the normal otocyst, with dominant-negative electroporated embryos averaging  $212 \pm 41$  μm ( $\pm$ s.e.m.;  $n=12$ ) in length along the rostrocaudal axis compared with  $301 \pm 30$  μm ( $n=8$ ) in controls. In some embryos, the vesicle lost the endolymphatic duct. In a few cases, we achieved unilateral electroporation. In these embryos, the electroporated side had a markedly smaller vesicle compared with a normal vesicle on the contralateral side (data not shown). In situ hybridization revealed that *Sox10* (Fig. 7C,D) retained its normal pattern (compare with Fig. 7A,B). *Notch* and *Lunatic fringe* also retained their normal pattern, though *Lunatic fringe* expression was lost in the smallest vesicles (data not shown).

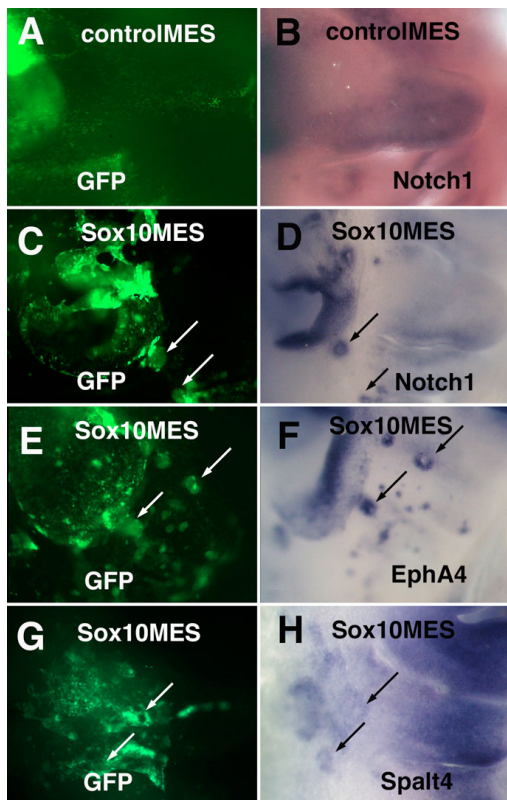
We next examined whether the changes in vesicle size were due to increased cell death and/or decreased proliferation. To this end, we performed TUNEL staining (Fig. 7E,F,G,H). At 8 hours after electroporation, we observed approximately twice as many TUNEL-positive cells in the ectoderm of dominant-negative electroporated embryos as in control electroporated embryos ( $9.4 \pm 5.4$ ;  $\pm$ s.e.m.,  $n=8$  TUNEL-positive cells per section compared with  $5.25 \pm 2.4$ ,  $n=8$  in controls,  $P < 0.05$ ). By contrast, no significant alterations in phosphohistone H3 levels ( $P < 0.4$ ) were noted between dominant-negative [ $n=5$ ;  $1.94 \pm 0.4$  ( $\pm$ s.e.m.) positive cells in the placode per section] and control ( $n=6$ ;  $1.71 \pm 0.18$  positive cells) embryos. These data suggest that the decrease in otic vesicle size caused by electroporation of truncated *Spalt4* may be due to an early increase in cell death but not to changes in cell proliferation.

In addition to the otic level, the truncated construct was electroporated at midbrain-rostral hindbrain level ectoderm, which does not normally express *Spalt4*. In these embryos, GFP-labeled cells migrated into the trigeminal ganglia in a manner similar to normal embryos (data not shown). This contrasts with embryos electroporated with full-length *Spalt4*, where GFP-positive cells remained in the ectoderm and contributed few or no cells to the trigeminal ganglion.

### Sox10 overexpression can induce ectopic vesicles

*Spalt4*-induced vesicles have ectopic *Sox10* expression. In a recent study in *Xenopus*, *Sox10* overexpression induced some vesicle-like structures in the vicinity of the ear (Taylor and Labonne, 2005). To test if a similar function was present in birds and if *Sox10* would phenocopy *Spalt4*, we electroporated a construct encoding *Sox10* (McKeown et al., 2005) into the cranial ectoderm. Similar to *Spalt4* overexpression, this generated multiple ectopic vesicles that expressed *Notch1* (Fig. 8C,D) and *EphA4* (Fig. 8E,F) suggesting that *Sox10* is epistatic to *Spalt4*. However, we did detect some weakly *Spalt4*-positive ectopic vesicles (Fig. 7G,H), suggesting a more





**Fig. 8. Mis-expression of *Sox10* generates ectopic vesicle that express otic vesicle genes.** (A) GFP fluorescence in an embryo electroporated with a control-GFP vector. (B) Embryo in A hybridized with *Notch1*. (C) An embryo electroporated with a *Sox10*-GFP construct shows GFP-positive ectopic vesicles (arrows). (D) Embryo in C hybridized with a *Notch1* probe. The ectopic vesicles (arrows) express *Notch1*. (E) An embryo electroporated with a *Sox10*-GFP construct shows GFP-positive ectopic vesicles (arrows). (F) Embryo in E hybridized with an *EphA4* probe. The ectopic vesicles (arrows) express *EphA4*. (G) An embryo electroporated with a *Sox10*-GFP construct shows GFP-positive ectopic vesicles (arrows). (H) Embryo in G hybridized with a *Spalt4* probe. The ectopic vesicles (arrows) express *Spalt4*.

complicated gene regulation. One difference between *Spalt4* and *Sox10* is that the latter generated ectopic vesicles adjacent to the trigeminal ganglia whereas *Spalt4* did not (data not shown).

## DISCUSSION

### ***Spalt4* is expressed early in placode development**

Placodes initially arise from a common preplacodal domain in the early embryo that subsequently becomes subdivided into individual placodes of either sensory (olfactory, lens, otic) or neurogenic (forming cranial sensory ganglia) character (Streit, 2004). Members of the *Six-Eya-Dach* pathway are expressed in a crescent-shaped domain of anterior ectoderm that is postulated to be of general placodal character (Brugmann and Moody, 2005; Streit, 2004). Later, individual placodes become distinguishable in the cranial ectoderm by thickening of the epithelium and expression of characteristic genes. For example, *Pax6* is expressed in lens and olfactory placodes (Bhattacharyya et al., 2004), *Pax3* in trigeminal placodes (Baker et al., 1999) and *Pax2* in otic and epibranchial placodes (Baker and Bronner-Fraser, 2001; Groves and Bronner-Fraser, 2000). No single gene imbues placodes with

a specific identity; instead, it is probable that co-expression of several genes is required for placode formation and differentiation.

In this study, we show that *Spalt4* expression overlaps that of *Six-Eya-Dach* genes in the preplacodal ectoderm, and is detected as early as stage 3 in the chick. *Spalt4* subsequently resolves to the presumptive otic, lens and olfactory placode regions by stage 10, concomitant with the time during which non-placodal ectoderm loses competence to form otic placode (Baker et al., 1999; Groves and Bronner-Fraser, 2000).

### ***Spalt4* expression causes the formation of ectopic pits**

One of the best-studied placodes is the otic placode, which forms the inner ear (Solomon et al., 2004; Streit, 2001). It initiates as a patch of thickened ectoderm on either side of the hindbrain that invaginates to form otic vesicles (otocysts). Subsequently, the otocyst becomes regionalized, giving rise to the complex inner ear including the cochlea, the different parts of the vestibular system and the endolymphatic duct. A number of different cell types originate from this epithelium, including mechanosensory hair cells, support cells and various other specialized cell types. Other cells delaminate from the placode and migrate next to the neural tube to form the acoustic ganglion. Signals from neighboring tissue, such as Fgf (Ladher et al., 2000; Ladher et al., 2005; Liu et al., 2003; Maroon et al., 2002; Vendrell et al., 2000) and Bmp4 (Chang et al., 1999; Gerlach et al., 2000; Merlo et al., 2002), appear to induce the otic placode and/or activate specific patterns of gene expression. We have shown that *Spalt4* is also induced in stage 4 ectoderm by Fgf2. Recent results have also implicated Wnt signaling in otic vesicle induction (Ladher et al., 2000; Ohyama et al., 2006).

Electroporation of *Spalt4* results in formation of small ectodermal pits near the otic vesicle or even laterally in the branchial arch ectoderm. Adjacent to the hindbrain, *Spalt4* induced ectopic vesicles that morphologically resemble otic vesicles and express otic markers. It is interesting to note that not all *Spalt4*-electroporated ectodermal cells invaginate. In almost all embryos, some remain in the ectoderm adjacent to the pits. Perhaps this reflects some intrinsic limits to ectopic vesicle size, density of electroporated cells, or requirement for additional signals in some cell populations. These results suggest that *Spalt4* alone is not sufficient to induce invagination in all ectoderm.

Ectopic vesicles express a number of genes characteristic of the otic vesicle and important for normal ear development; these include *Notch* (Adam et al., 1998), *Lunatic fringe* (Cole et al., 2000), *Bmp4* (Cole et al., 2000), *Dlx3* (Pera et al., 1999), *Dlx5* (Streit, 2002), *Sox8* (Bell et al., 2000), *Sox10* (Cheng et al., 2000), *Tbx1* (Chapman et al., 1996), *Tbx3* (Chapman et al., 1996) and *Nkx5.1* (Herbrand et al., 1998).

*Spalt4* is sufficient to recapitulate some but not all of the molecular events necessary for normal ear development. For example, we were unable to detect the expression of many genes in the *Six-Dach-Eya* pathway. One possibility is that the *Six-Eya-Dach* pathway is upstream of *Spalt4*. Another possibility is that regulation of this pathway may involve a gene network independent and perhaps parallel to that induced by *Spalt4*. *Six1* and *Eya1* mutants have been shown to have poorly developed auditory systems (Li et al., 2003; Ozaki et al., 2004; Xu et al., 1999; Zheng et al., 2003). Though the ears progress to the otic vesicle stage, they fail to form middle and inner ear structures or neurons, as if stalled at the vesicle stage rather than differentiating further (Zheng et al., 2003). Similarly, we were unable to detect neurons in the ectopic vesicles

72 hours after *Spalt4* electroporation. An intriguing possible reason why *Spalt4*-induced vesicles do not progress beyond the otic vesicle stage and fail to generate neurons is because of their failure to upregulate *Six1* and *Eya1*. Alternatively, the *Six-Eya-Dach* genes may require region-specific signals that are absent at the sites where ectopic vesicles form. The latter possibility seems likely in the case of *Dach2*, since it was expressed only in ectopic vesicles next to the hindbrain. *Tbx1* and *Tbx3* are also influenced by other factors since they were expressed only in ectopic vesicles caudal to the endogenous otic vesicle.

Recent studies have highlighted interesting similarities between the vertebrate inner ear and Johnston's organ in *Drosophila* (Boekhoff-Falk, 2005). Many of the genes necessary for specification or function of the auditory cells in *Drosophila* are also required in the vertebrate inner ear. In *Drosophila spalt* and *spalt-related* are required for the formation of Johnston's organ (Dong et al., 2003). However, the roles of *spalt* genes in vertebrate and fly auditory development may not be completely analogous. In *Drosophila*, *spalt* has been shown to be downstream of *distalless* (Dong et al., 2002). By contrast, we find that chick *Dlx* genes are upregulated by misexpression of chick *Spalt4*. Furthermore, *Dlx3* or *Dlx5* overexpression fails to induce *Spalt4* expression (data not shown). The Iroquois homologues *Irx1* and *Irx2* are upregulated by *Spalt4* (Table 1), though they are repressed by *spalt* in *Drosophila* wing development (de Celis and Barrio, 2000).

Ectopic Fgf2, Fgf3 or Fgf8 induce ectopic vesicles that express otic markers such as *Notch1*, *Pax2* and *Nkx5.1* (Adamska et al., 2000; Vendrell et al., 2000). In addition to Fgf, other signals may be involved in regulation of *Spalt4* and/or other placodal determinants. Vitamin A-deficient chick embryos lack posterior hindbrain, but develop ectopic *Pax2*-positive vesicles (Kil et al., 2005). Overexpression in *Xenopus* of the secreted phospholipase *Rossy* induces ectopic olfactory vesicles, and, by microarray analysis, has been shown to upregulate a member of the *spalt* family, *Xspalt1* (Munoz-Sanjuan and Brivanlou, 2005). Overexpression of other transcription factors has been shown to result in ectopic vesicles in other species. *Pax6* overexpression generates ectopic lens vesicles (Altmann et al., 1997) and *Sox10* can generate ectopic otic vesicles (Taylor and Labonne, 2005). Ectopic *Six3* expression in mice leads to ectopic vesicle formation near the otocyst (Lagutin et al., 2001), and injection of *Sox3* (Koster et al., 2000) in medaka gives rise to vesicles of either otic or lens character. However, we failed to detect upregulation of either *Six3* or *Sox3* after misexpression of *Spalt4*; this could reflect species differences or a lack of epistatic interactions between these transcription factors. It is currently unclear if any of these factors directly regulates *Spalt4* in the chick. Interestingly, constitutively active Notch has been shown to cause the formation of ectopic structures expressing ear-specific genes, consistent with the possibility that *Spalt4* is upstream of *Notch* in this cascade (Daudet and Lewis, 2005). We have found that ectopic *Sox10* expression can generate ectopic vesicles in the chick, similar to results previously described in *Xenopus* (Taylor and Labonne, 2005). These ectopic vesicles also express *Spalt4*. The exact relationship between these two genes has yet to be determined, but may involve a feedback loop. One difference is that *Spalt4* was unable to generate ectopic vesicles expressing otic-specific genes at the level of the trigeminal ganglia, whereas *Sox10* was able to do so. This indicates that *Spalt4* and *Sox10* respond differently to the signals in the ectoderm at midbrain level and implies that *Sox10* may act downstream of *Spalt4*.

Overexpression of *Spalt4* within the otic vesicle itself causes alterations in morphology and patterns of gene expression in the developing ear. Defects include formation of multiple vesicles resembling otic vesicles, and the failure to form a closed otic vesicle. In both cases, *Pax2*, *Lunatic fringe* and *Notch1* are expressed in a non-regionalized fashion. In the most extreme cases where the otic vesicle fails to close, *Sox10* is expressed throughout the otic ectoderm rather than being confined to the lateral half as in control electroporated embryos. Normally, the expression of *Spalt4* in the closed vesicle begins to be downregulated at stage 16. However, in electroporated embryos, *Spalt4* expression is maintained. This may lead to altered expression of other genes, the loss of regionalizing signals and the observed abnormalities in the otic vesicle. Interestingly, activation of canonical Wnt signaling as well as Fgf overexpression in the ear also leads to formation of open, oversized ears (Ladher et al., 2000; Ohyama et al., 2006; Vendrell et al., 2000).

Expression of *Spalt4* at the midbrain level interfered with the normal ingress of placode cells into the trigeminal ganglia. Few GFP cells contributed to the ganglia and the number of placode-derived cells was also reduced, resulting in a malformed ganglia. A similar effect was seen in the failure of *Spalt4*-expressing neural crest cells to contribute to the trigeminal ganglia (Barembaum and Bronner-Fraser, 2004). Furthermore, we were unable to detect *Pax3* in the GFP-expressing thickened ectoderm (data not shown). At epibranchial placode levels, *Spalt4*-electroporated cells also formed ectopic vesicles and failed to contribute to the ganglia derived from the epibranchial placodes after 48 hours. Since ectopic vesicles were found in ectoderm that would normally give rise to neurons, this probably reflects a change in cell fate from neurogenic to sensory. The cell fate switch is reminiscent of the activity of *spalt* in *Drosophila* where it affects fate determination in a number of different lineages (de Celis et al., 1996; Elstob et al., 2001; Rusten et al., 2001).

### Reduction of *Spalt4* activity causes abnormalities in ear development

Our results show that *Spalt4* is not only sufficient for vesicle formation but also necessary for proper otic development. Introduction of a truncated, dominant-negative *Spalt4* results in abnormal otic vesicles that are drastically reduced in size. In general, otic gene expression remains the same and vesicles retain a regionalized pattern. The reduction in vesicle size appears to be caused by increased cell death, as assayed by TUNEL. That otic vesicles do form in the presence of the dominant-negative *Spalt4*, albeit reduced in size, may indicate that dominant-negative *Spalt4* may not fully abrogate endogenous *Spalt4* activity. *Spalt4* is normally expressed well before the time that we introduce the dominant-negative construct. Thus, it is likely that we do not achieve full knockdown of transcription factor activity. Also we cannot rule out the possibility that other genes may be acting on the ectoderm to partially compensate for the loss of *Spalt4*. It is also worth noting that whereas *Sox10* can be induced in ectopic vesicles, it is not reduced in otic vesicles electroporated with the dominant-negative construct. A possible explanation is that *Sox10* may not be directly induced by *Spalt4*.

Consistent with our observations in chick, humans with Okihoro syndrome, in which *SALL4* is mutated, have hearing defects as well as abnormalities of the heart, kidney and limbs (Kohlhase et al., 2005). By contrast, no hearing defects have been reported in heterozygous mutant mice (Sakaki-Yumoto et al., 2006), though some hearing defects have been detected in mice with a truncated *Spalt4* (Warren et al., 2007).



## Conclusions

The finding that chick *Spalt4* is expressed earlier than otic placode markers such as *Pax2*, *Dlx3* and *Dlx5* in the otic placode raises the intriguing possibility that *Spalt4* may have a role in establishing the placode domain. *Spalt4* alone, however, is not sufficient for normal otic vesicle formation since it fails to form ectopic vesicles of normal size that express all ear markers. It is more likely that *Spalt4* is an important component in the multiple steps leading to the formation of the ear. Our functional analysis suggests that it may initiate the process of invagination in the early ectoderm in response to region-specific signals along the rostrocaudal axis and to upregulate appropriate gene expression, such as ear-specific genes, in the vesicles in the hindbrain region.

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