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The mouse *Foxi3* transcription factor is necessary for the development of posterior placodes

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ABSTRACT

The inner ear develops from the otic placode, one of the cranial placodes that arise from a region of ectoderm adjacent to the anterior neural plate called the pre-placodal domain. We have identified a Forkhead family transcription factor, *Foxi3*, that is expressed in the pre-placodal domain and down-regulated when the otic placode is induced. We now show that *Foxi3* mutant mice do not form otic placodes as evidenced by expression changes in early molecular markers and the lack of thickened placodal ectoderm, an otic cup or otocyst. Some preplacodal genes downstream of *Foxi3-Gata3, Six1* and *Eya1*-are not expressed in the ectoderm of *Foxi3* mutant mice, and the ectoderm exhibits signs of increased apoptosis. We also show that Fgf signals from the hindbrain and cranial mesoderm, which are necessary for otic placode induction, are received by pre-placodal ectoderm in *Foxi3* mutants, but do not initiate otic induction. Finally, we show that the epibranchial placodes that develop in close proximity to the otic placode and the mandibular division of the trigeminal ganglion are missing in *Foxi3* mutants. Our data suggest that *Foxi3* is necessary to prime pre-placodal ectoderm for the correct interpretation of inductive signals for the otic and epibranchial placodes.

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1. Introduction

The inner ear arises from a population of thickened ectodermal cells adjacent to the hindbrain, termed the otic placode (Groves, 2005). It forms during the differentiation of the neural plate border region through a series of inductive events occurring at the end of gastrulation. The border between neural and non-neural ectoderm contains two populations of cells that are molecularly distinct from the neural plate and future epidermis: the presumptive neural crest and the pre-placodal region (Grocott et al., 2012; Groves and LaBonne, 2014; Milet and Monsoro-Burq, 2012). Pre-placodal ectoderm is induced by a combination of FGF signaling and the attenuation of Wnt and BMP signals (Bailey and Streit, 2006; Grocott et al., 2012; Litsiou et al., 2005; Streit, 2007). Once induced, a second set of signals direct different regions of pre-placodal ectoderm to differentiate into distinct cranial placodes along the anterior–posterior axis of the head (Baker and Bronner-Fraser, 2001; Jidigam and Gunhaga, 2013; Patthey and Gunhaga, 2011, 2013; Schlosser, 2006, 2010). These cranial placodes give rise to paired sensory organs of the vertebrate head, including the entire inner ear and the VIIIth cranial ganglion that innervates the sensory regions of the ear.

The division of embryonic ectoderm into neural plate, neural crest, pre-placodal region and epidermis is achieved in part by the establishment of different domains of transcription factors (Grocott et al., 2012; Groves and LaBonne, 2014). For example, non-neural ectoderm initially expresses *Dlx5/6*, *Ap1*, *Gata2/3* and *Foxi* genes, which mutually reinforce each other's expression and inhibit neural transcription factors *Sox2* and *Sox3* (Grocott et al., 2012). As the pre-placodal region is induced, *Dlx5/6*, *Ap1* and *Gata2/3* become restricted to this region, where they are co-expressed with definitive markers of the pre-placodal region *Six1* and *Six4,* and their co-factors *Eya1* and *Eya2* (Christophorou et al., 2009; McLarren et al., 2003; Streit, 2001, 2007). A posterior group of pre-placodal cells responds to FGF signaling from the adjacent

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hindbrain or cranial mesoderm and differentiates into the otic placode (Ohyama et al., 2007). FGF signaling is necessary for otic placode induction in all vertebrates examined to date, and application of FGF to pre-placodal tissue is sufficient to initiate the expression of many, but not all, otic placode genes (Ladher et al., 2000, 2005; Leger and Brand, 2002; Liu et al., 2003; Maroon et al., 2002; Martin and Groves, 2006; Phillips et al., 2001; Urness et al., 2010; Wright and Mansour, 2003).

We have previously shown that competence to respond to FGF signals correlates with pre-placodal identity (Martin and Groves, 2006). However, the molecular basis of this competence and how it is established are still not clear. We have found that *Foxi3*, a member the Forkhead transcription factor family, is expressed in the pre-placodal region and is sufficient to induce a number of pre-placodal and non-neural ectoderm genes (Khatri et al., 2014; Khatri and Groves, 2013; Ohyama and Groves, 2004a). Knockdown of *Foxi3* in chick embryos, or *foxi1* in zebrafish, causes a failure of otic placode induction (Hans et al., 2007; Hans et al., 2013; Khatri et al., 2014; Nissen et al., 2003; Solomon et al., 2003). However, technical limitations of targeting the presumptive otic placode at early stages and the failure of morpholinos to produce a complete loss of function in chick, led us to generate a mouse knockout of *Foxi3* (Edlund et al., 2014). Here, we show that *Foxi3* mutant mice completely lack an inner ear and show no evidence of expression of any otic placode markers. We show that three transcription factors downstream of *Foxi3*, *Gata3*, *Eya1* and *Six1,* are absent in *Foxi3* mutants. We also show that the pre-placodal region in *Foxi3* mutant tissue received FGF signaling, but that this tissue fails to execute a program of otic placode differentiation. Moreover, the neurogenic epibranchial and trigeminal placodes are either disrupted or completely absent in *Foxi3* mutant mice. Together, our data suggest that *Foxi3* acts at multiple stages of otic placode induction and may function as a pioneer factor necessary for pre-placodal ectoderm to execute an inner ear program in response to FGF signaling.

2. Materials and methods

2.1. Mouse line maintenance and genotyping

The generation of *Foxi3* mutant mice was recently described (Edlund et al., 2014). The *Foxi3* deletion allele (*Foxi3*-del) was maintained by breeding heterozygous mice. Primers used to genotype embryos were f3G1 (5′-GGC CTT GTC TCA ACC AAC AG-3′), f3G2 (5′-GTT TCC TGT ATC CCT GGC TG-3′) and f3G3 (5′-CTT GGA ATG GGT TGA CTG AG-3′). f3G1 and f3G2 produce a 350 bp band corresponding to the wild-type allele and f3G1 and f3G3 yield a 600 bp band corresponding to the foxi3-del allele. *Foxi2* mice were bred as homozygotes similarly to the *Foxi3* line. The primers used for genotyping were as follows: f2G1 (5′-TGG TTA GCT CAG TTC CAC TG-3′), f2G2: (5′-ATT GAT TCC ACT GGT CCC TG-3′) and f2G3: (5′-TGC CTC CCC TCC AAA TAT TCA C-3′). G1 and G2 were used for wild-type band of 220 bp whereas G1 and G3 resulted in a 530 bp deletion band. *Foxi2/3* double mutant embryos were generated by breeding *Foxi2*$^{-/-}$;*Foxi3*$^{+/-}$ mice.

2.1.1. Generation of Foxi2 mutant mice

A targeting vector for the mouse *Foxi2*-floxed-neo allele was constructed using BAC recombineering (Warming et al., 2005). Briefly, an approximately 12.4 kb genomic DNA fragment containing exon 2 of mouse *Foxi2* was retrieved from a BAC clone bMQ303G3 of a 129Sv BAC genomic library obtained from the Wellcome Trust Sanger Institute (Adams et al., 2005). Using recombineering, a loxP site was inserted upstream of exon 2, and an Frt-PGKNeo-Frt-LoxP sequence was inserted downstream of exon 2 (Meyers et al., 1998). Electroporation of the targeting vector into ES cells, screening of the targeted ES cells and blastocyst injection were performed by the transgenic core facility at Norris Cancer Center of the University of Southern California. Germline *Foxi2*-floxed-neo founder mice were identified and confirmed by genomic Southern blotting to detect the extra EcoRV and NheI sites introduced by the Frt-PGKNeo-Frt-LoxP sequence. The *Foxi2-del* allele used in this study was generated by crossing the *Foxi2-floxed-neo* allele with a *CMV-Cre* line (JAX Mice, stock #003465).

2.1.2. Probe synthesis and in situ hybridization

Digoxygenin-labeled RNA probes for whole mount in situ hybridization were synthesized from cDNA clones using standard techniques (Stern, 1998). Whole mount in situ hybridization was performed using a protocol modified from Domingos Henrique (Henrique et al., 1995) as previously described (Khatri et al., 2014). cDNA plasmids were kindly provided by the following individuals: *Dlx5* (Jin-Xian Lie), *Gata3* (Doug Engel), *Six1*, *Eya1* and *Eya2* (Pin-Xian Xu), *Six4* (Pascal Maire), *Sox9* (Andreas Schedl), *Pax2* (Gregory Dressler), *Pax8* (Meinrad Busslinger), *Neurog1*, *Neuro2*, *NeuroD* (Qiufu Ma), *Erm* (Katherine Shim), *Fgf3, Fgf10*, and *Fgfr2* (Suzi Mansour), and *Spry2* (Gail Martin). For *Foxi3* in situ hybridization, we used a cDNA probe for exon 2 of mouse *Foxi3* (Ohyama and Groves, 2004a). For sectioning, whole mount in situ specimens were transferred to 15% sucrose in PBS for equilibration and embedded in 7.5% gelatin and 15% sucrose in PBS. Frozen embryos were sectioned transversely at 14 μm with a Leica cryostat and collected onto Superfrost Plus slides. After drying overnight and mounting in glycerol, they were visualized with an upright microscope and digitally photographed.

2.2. Immunohistochemistry

Embryos were fixed and embedded in gelatin (7.5% gelatin, 15% sucrose in PBS). 14 μm thick sections were collected on Superfrost Plus slides. Antibodies to activated Caspase-3 (AF835, R&D Systems) and class III Beta-Tubulin (Tuj1; MMS-435P, Covance) were diluted 1:200 and 1:500, respectively, in blocking buffer (PBS with 0.1% Triton X-100 and 10% goat serum) and applied to sections overnight at 4 °C. Secondary antibodies (AlexaFluor 488 goat anti-rabbit for Casp3 and AlexaFluor 594 goat anti-mouse for Tuj1) were diluted 1:1000 in blocking buffer and applied to sections for one hour at room temperature. After washing, sections were counterstained with DAPI (10 μg/ml).

2.2.1. Statistical analysis of Casp3+ cells

Transverse 14 μm sections were collected from *Foxi3* mutant and wild-type embryos from the anterior-most region of the head to the level of the first somite and were stained with antibodies to activated Caspase-3 and counterstained with DAPI (10 μg/ml). Apoptotic cells that are normally observed in the anterior and ventral regions of the developing forebrain of wild-type embryos were used as a positive control. Casp3+ cells in the ectodermal layer adjacent to the neural plate were counted between the posterior forebrain and first somite, as shown in the schematic diagram in Fig. 4C. Numbers of apoptotic cells were compared between mutant and wild-type embryos using a paired two sample *t*-test.

3. Results

3.1. Foxi3 is expressed in mouse pre-placodal ectoderm and is down-regulated as the otic placode is induced

We previously reported that *Foxi3* has a dynamic expression

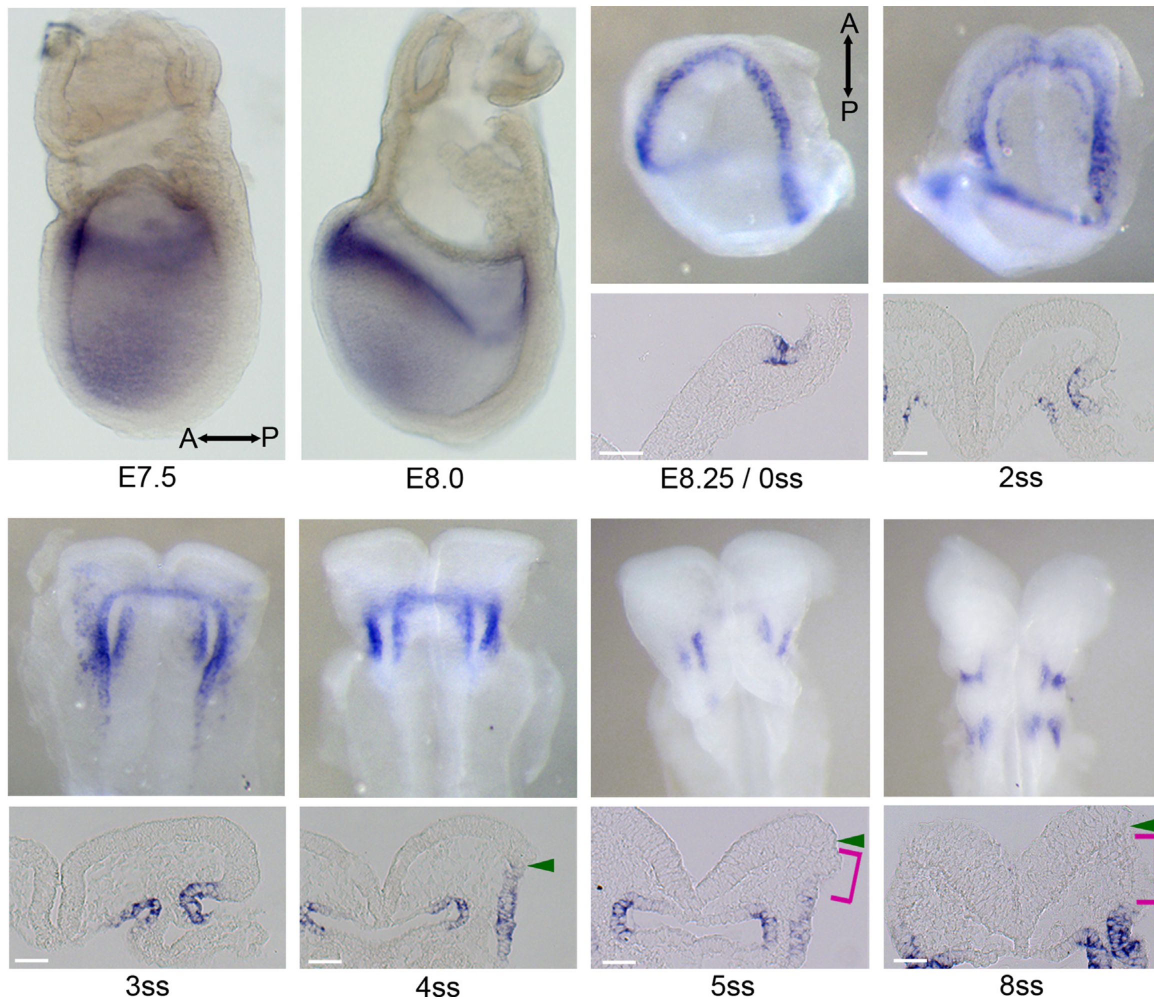

Fig. 1. Dynamic expression of *Foxi3* in embryonic ectoderm between stages E6.5 and E8.5. *Foxi3* expression is observed in the most anterior region of the epiblast at E7.5 and is slowly refined to non-neural ectoderm by E8.0. At the 0 somite/headfold stage (E8.25/0ss) it is only expressed in non-neural ectoderm at the neural plate border and is absent from all other regions of the embryo. Over the next 12 hours, *Foxi3* gradually refines to pre-placodal ectoderm and pharyngeal endoderm at the level of the future midbrain and hindbrain. By the 3-5ss, *Foxi3* expression is restricted to the posterior pre-placodal ectoderm and pharyngeal endoderm. After otic placode induction, *Foxi3* is down-regulated from dorsal pre-placodal ectoderm (magenta brackets) and is only found in ventral parts of the head ectoderm. ss – number of pairs of somites. Green arrowheads mark the neural plate-epidermis boundary. Scale bar = 150 μm.

profile in pre-placodal ectoderm and branchial arch ectoderm and endoderm between E8.0 and E9.5 (Edlund et al., 2014; Ohyama and Groves, 2004a). To obtain a more detailed picture of the changing pattern of *Foxi3* expression during establishment of the neural plate border and induction of the otic placode, we performed in situ hybridization for *Foxi3* from E6.5 to E8.5 (Fig. 1). We first detected *Foxi3* expression in the anterior epiblast at E7.5, which was then refined to the neural plate border shortly before the first somites appear at about E8.25 (Fig. 1). As the first three pairs of somites condense over the next six hours, *Foxi3* is rapidly restricted to the anterior region of the embryo. Sections of embryos at these ages reveal *Foxi3* expression in the pre-placodal region at the edge of the thickened neural plate as it transitions from non-neural ectoderm, similar to what has been observed in chick (Khatri and Groves, 2013). At the 2 somite stage (2ss), we observed faint *Foxi3* expression in pharyngeal endoderm, and this expression strengthened and became restricted to the most lateral out-pocketing of the endoderm over the next few hours (Fig. 1). By the 4ss, *Foxi3* expression is down-regulated from the anterior pre-placodal region and becomes restricted to the posterior pre-placodal region and the endoderm beneath it. At this point (4-5ss), the boundary between the neural plate and pre-placodal ectoderm can clearly be seen on sections. *Foxi3* is expressed in ectoderm that is starting to thicken to form the otic placode. From the 5ss onwards, *Foxi3* is down-regulated in the otic placode region (Fig. 1, magenta brackets) and is restricted to the future branchial arch ectoderm and pouch endoderm, beginning in the future first arch at the 5ss. A second patch of arch and pouch expression is apparent at the 8ss (Fig. 1; (Edlund et al., 2014; Ohyama and Groves, 2004a)). In summary, *Foxi3* is expressed ubiquitously in the epiblast at E7.5, becomes restricted first to non-neural ectoderm along the entire body axis by E8.0 and then to the pre-placodal region and future otic placode as the first somites are added, before disappearing from the otic placode by the 8ss.

3.2. The otic placode is not induced in Foxi3 mutant mice

We recently showed that *Foxi3* mutant mice lack an external and middle ear and show no evidence of a differentiated inner ear or temporal bone (Edlund et al., 2014). To determine the stage at which inner ear development fails in *Foxi3* mutants, we examined early markers of the otic placode and otocyst. *Pax2* is the earliest marker of the otic placode in mouse (Ohyama and Groves, 2004b; Urness et al., 2010; Wright and Mansour, 2003). It appears as a faint streak of ectodermal staining contiguous and posterior to the broad domain of *Pax2* expression in the future midbrain and

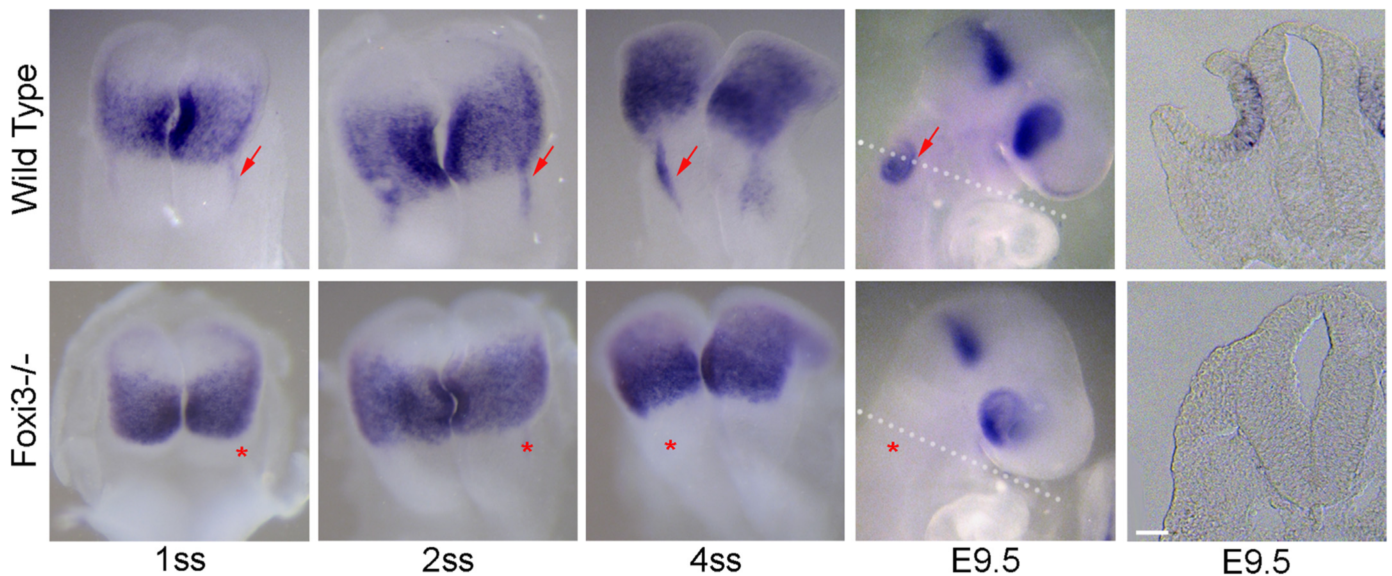

Fig. 2. Pax2, the earliest marker of the otic placode, is absent in *Foxi3* mutants. *Pax2* is expressed in the midbrain–hindbrain region of wild-type and *Foxi3* mutant embryos and in the developing otic region of wild-type embryos (red arrows). However, *Pax2* is absent from the otic region of *Foxi3* mutants (asterisks). Number of wild type embryos examined: 2 (1ss), 3 (2ss), 4 (4ss), 20 (E9.5). Number of mutant embryos examined: 5 (1ss), 4 (2ss), 4 (4ss), 10 (E9.5). At E9.5, *Pax2* expression persists in the wild-type otic cup, but neither *Pax2* expression nor an otic cup is visible in *Foxi3* mutants. The dotted lines indicate the approximate plane of section in the far right panels. ss – numbers of pairs of somites. Scale bar = 150 μm.

anterior hindbrain (Fig. 2; Ohyama and Groves, 2004b), before enlarging into a distinct otic placodal domain on either side of the posterior hindbrain. *Pax2* expression remains strong in the invaginating otic cup (Fig. 2; Ohyama and Groves, 2004b; Urness et al., 2010; Wright and Mansour, 2003). We saw no evidence for *Pax2* expression in this region in *Foxi3* mutants at any stage examined, although *Pax2* expression in the future midbrain–hindbrain region was not affected (Fig. 2). We next examined *Foxi3* mutant embryos at the otic cup stage to determine whether any morphological or molecular evidence of the otic placode persisted in these mutants. All otic markers tested (*Pax8, Dlx5, Sox9, Foxg1*) were absent from Foxi3 mutant embryos (Fig. 3). Moreover, we saw no evidence for placode-like thickening or invagination of ectoderm adjacent to the hindbrain. In approximately 10% of mutant embryos, we saw a tiny ball of epithelial cells immediately adjacent to the hindbrain that expressed otic markers such as *Pax8* or *Dlx5* (data not shown). These small structures were not observed in embryos at stages older than E9.5.

To investigate the identity of *Foxi3* mutant cells in the region of the posterior hindbrain, we examined the expression of *Foxi2*. *Foxi2* marks the epidermal cells surrounding the otic placode region, but it is completely absent from the otic placode in wild-type embryos ((Khatri and Groves, 2013; Ohyama and Groves, 2004a; Ohyama et al., 2006); Fig. 4A). In *Foxi3* mutants, however, this region was covered with *Foxi2*-expressing cells, suggesting that there are no otic placode cells present. To determine whether the absence of the otic placode and otic cup in *Foxi3* mutant embryos could be partially explained by the death of differentiating otic placode tissue, we examined activated Caspase-3 in wild-type and *Foxi3* mutant embryos between the 4-8ss (Fig. 4B). We observed some apoptotic cells around the pre-placodal region in *Foxi3* mutant embryos (Fig. 4B, white arrows). There was a significant difference in the total number of Casp3+ cells in the posterior pre-placodal region in mutant embryos compared to wild-type embryos (Fig. 4C and D; 4 ± 1 (wild type) versus 49 ± 14 (mutant), $p < 0.05$). This suggests that there is significant cell death in the mutant pre-placodal region, especially where the future otic and neurogenic placodes will form.

3.3. *Foxi3 is necessary for the expression of a subset of non-neural and pre-placodal ectoderm genes*

Our data suggest that the induction of the otic placode and formation of the otocyst fail to occur in *Foxi3* mutant mice. We recently observed a similar lack of early otic markers in chicken embryos after morpholino knockdown of *Foxi3* at late gastrula stages (Khatri et al., 2014). The complete lack of otic placode genes in *Foxi3* mutant mice is more severe than the changes seen in *foxi1* mutant or morphant zebrafish (Hans et al., 2013; Kwon et al., 2010; Nissen et al., 2003; Solomon et al., 2003). However, since *Foxi3* expression commences prior to the induction of the otic placode and decreases as the otic placode differentiates, the loss of *Foxi3* may be affecting the segregation of non-neural from neural ectoderm or the induction of the pre-placodal domain. We therefore used a panel of non-neural ectoderm and pre-placodal genes to determine how loss of *Foxi3* affected the development of the neural plate border and pre-placodal domain.

An early decision in the induction of the nervous system is the division of embryonic ectoderm into neural and non-neural ectoderm (Groves and LaBonne, 2014; Streit, 2007). The transcription factors *Dlx5, Dlx6* and *Gata3* are initially expressed in non-neural ectoderm and are later restricted to the pre-placodal region and ultimately the otic placode (Brown et al., 2005; Groves and Bronner-Fraser, 2000; Groves and LaBonne, 2014; Kwon et al., 2010; Lillevali et al., 2006; Sheng and Stern, 1999; Streit, 2007; Zheng et al., 2003). We observed no differences in the expression of *Dlx5* in pre-placodal ectoderm of *Foxi3* mutant embryos compared to wild type at E8.0 (Fig. 5). In contrast, *Gata3* was absent from the pre-placodal region in *Foxi3* mutant embryos, although we still observed *Gata3* signal in pharyngeal endoderm at these ages (Fig. 5). Mouse *Gata3* appeared at the headfold stage (3-4ss), after Dlx5 and Foxi3 were expressed, which is later than reported in zebrafish and chick ((Bhat et al., 2013; Khatri et al., 2014; Kwon et al., 2010); Figs. S1 and S2).

Shortly after the segregation of neural and non-neural ectoderm, a region of cells at the neural plate border forms the preplacodal region (Ahrens and Schlosser, 2005; Bhat et al., 2013; Brugmann et al., 2004; Kwon et al., 2010; Litsiou et al., 2005) and acquires a molecularly distinct identity defined by expression of

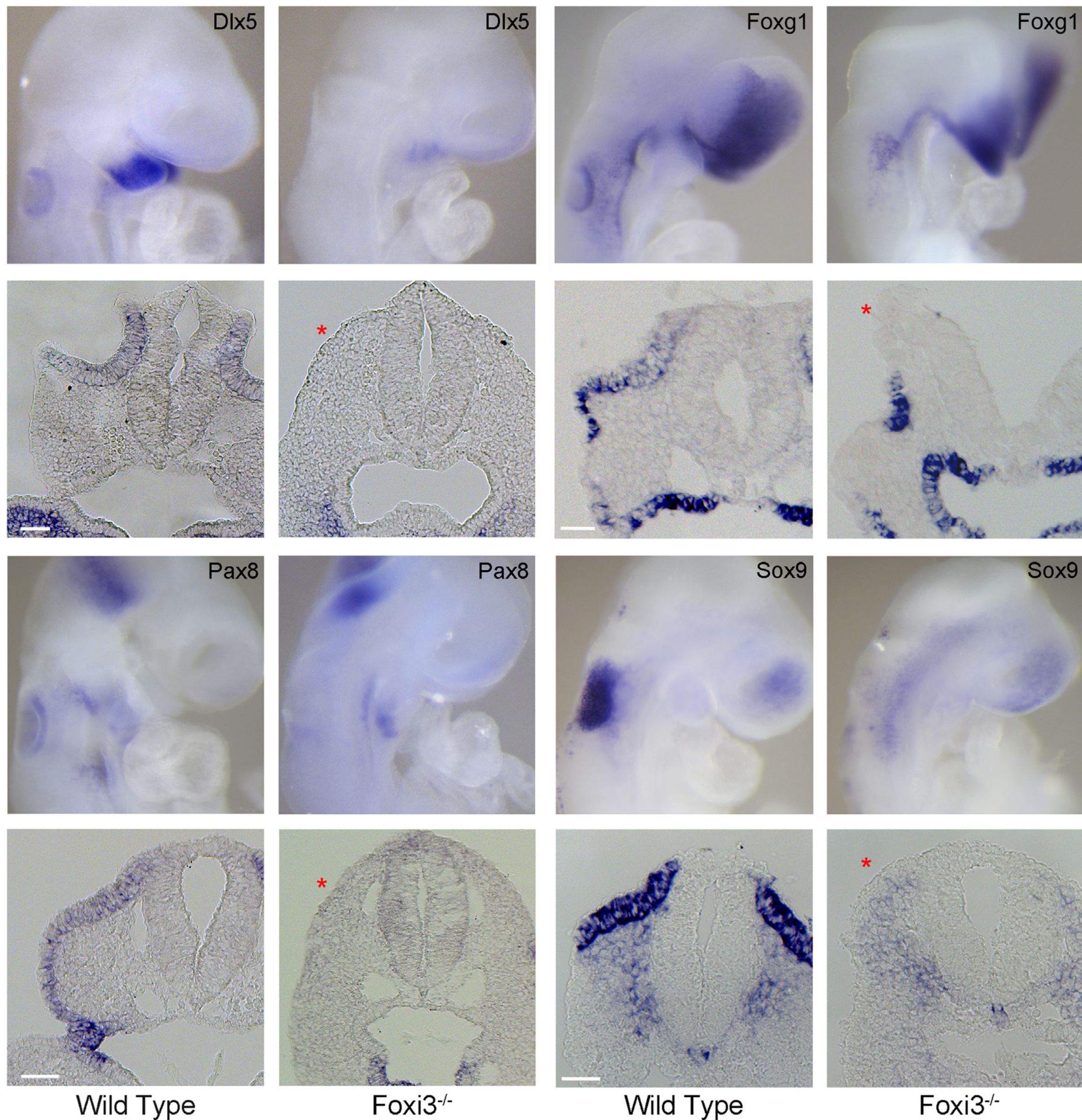

Fig. 3. *Foxi3* mutants do not express markers of the otic cup. Four genes that mark the otic placode, *Dlx5*, *Foxg1*, *Pax8* and *Sox9*, were examined in wild-type and *Foxi3* mutant embryos at the otic cup stage (E9.0-9.5). *Foxi3* mutants lack an otic cup or thickened invaginating placodal ectoderm (asterisks) and none of the four otic genes are expressed in ectoderm adjacent to the hindbrain, which normally differentiates into the otic placode. However, all four markers are expressed normally in other tissues with the exception of the pharyngeal arch region, which is disrupted in *Foxi3* mutants (Edlund et al., 2014). 5–6 wild-type or mutant embryos were examined for each marker. Scale bar = 150 μm.

Six and *Eya* family genes (Grocott et al., 2012; Groves and LaBonne, 2014; Streit, 2007). We next examined the expression of *Six1, Six4, Eya1* and *Eya2* in 3-4ss *Foxi3* mutant and wild-type embryos. *Eya2* and *Six4* were expressed normally in the pre-placodal region, cranial mesoderm and endoderm of *Foxi3* mutants (Fig. 6). In contrast, *Six1* and *Eya1* were not expressed in pre-placodal ectoderm of *Foxi3* mutants, although we continued to see robust expression in cranial mesoderm (*Six1* and *Eya1*) and endoderm (*Six1*; Fig. 6). Together, these results suggest that *Foxi3* regulates the expression of some non-neural ectoderm and pre-placodal genes (*Gata3, Six1, Eya1*) but not others (*Dlx5, Six4, Eya2*).

3.4. Foxi3 is not necessary for expression of FGF signaling pathway components or reception of FGF signals during otic placode induction

Many studies in multiple species have confirmed the essential role for FGF signaling in otic placode induction (reviewed in (Groves, 2005; Groves and Fekete, 2012)). In mice, the sequential actions of FGF8 in embryonic endoderm followed by FGF10 in cranial mesoderm and FGF3 in the hindbrain induce the otic placode from pre-placodal ectoderm (Ladher et al., 2005; Schimmang, 2007; Urness et al., 2010; Wright and Mansour, 2003). Since at least some pre-placodal markers are expressed in *Foxi3* mutant embryos (Fig. 6), we next tested whether this ectoderm remained competent to respond to FGF signaling. First, we examined expression of the FGF ligands *Fgf3* and *Fgf10.* Both ligands are expressed normally in the hindbrain (*Fgf3*) and cranial mesoderm (*Fgf10*) of *Foxi3* mutants (Fig. 7A). The FGF receptor *2* (*Fgfr2*) is expressed in the neural plate and non-neural ectoderm, and at lower levels in mesoderm and endoderm (Fig. 7B). *Fgfr2* expression was still present in *Foxi3* mutants in the pre-placodal region, although the intensity of the in situ hybridization signal in this region was somewhat lower than in controls. We saw no significant differences in *Fgfr1* expression between *Foxi3* mutant and

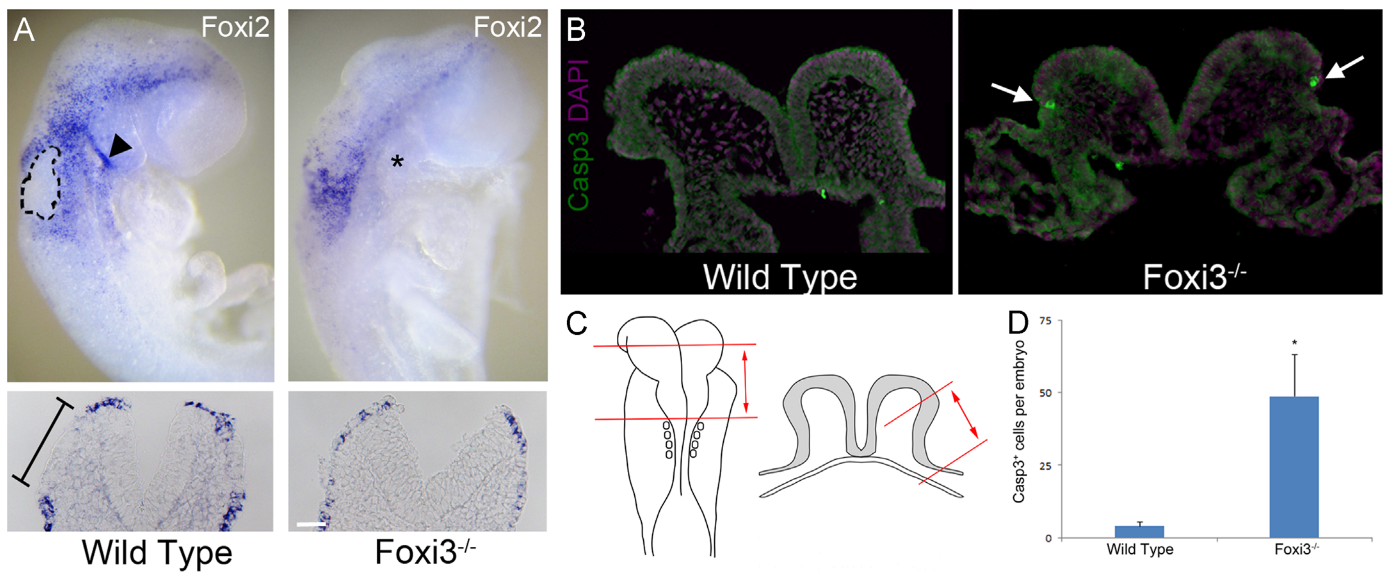

Fig. 4. Expression of epidermal and apoptotic markers in *Foxi3* mutants. (A) *Foxi2* is a marker that is normally absent in otic tissue (dotted area; brackets on section) and strongly expressed in the first pharyngeal cleft (black arrowhead). The *Foxi2*-negative otic region is absent in *Foxi3* mutants, and instead, *Foxi2*-expressing epidermis covers this region. The first pharyngeal arch is also disrupted in *Foxi3* mutants (asterisk; Edlund et al., 2014). 10 wild-type and 7 mutant embryos were examined... (B) Wild-type and *Foxi3* mutant embryos between 4-8ss were stained with an activated Caspase-3 antibody (green) to reveal apoptotic cells and counterstained with DAPI (magenta) to reveal nuclei. White arrows indicate the presence of apoptotic cells in the pre-placodal region. (C) Schematic diagram of a 4-8ss embryo and a transverse section to show the regions of the embryos that were quantified for Casp3+ cells in the anterior–posterior and medial-lateral axes (red lines). (D) Quantification of apoptotic cells in wild-type and *Foxi3* mutant embryos. Standard error is shown. $n=7$ for both wild-type and mutant embryos ($p<0.05$). Scale bar = 150 μm.

wild-type embryos (Fig. S3). Finally, we examined expression of several downstream markers of FGF signaling. Induction of Ets family transcription factors is commonly seen during FGF-mediated developmental events (Firnberg and Neubüser, 2002; Tsang and Dawid, 2004), including otic placode induction (Leger and Brand, 2002; Urness et al., 2010; Yang et al., 2013). Reception of FGF signaling also leads to the up-regulation of negative regulators of receptor tyrosine kinases, such as members of the Sprouty gene family (Cabrita and Christofori, 2008; Mahoney Rogers et al., 2011; Urness et al., 2010). To test whether FGFs induced downstream effectors of the FGF pathway in *Foxi3* mutant embryos, we examined expression of the Ets factor *Erm* and of *Spry2* in the placodal region. In both cases we saw no difference between wild-type and mutant embryos (Fig. 7C). Taken together, our data suggest that the FGF signaling pathway remains intact in *Foxi3* mutant embryos and that cells in the pre-placodal ectoderm are capable of responding to FGF signaling by up-regulating known FGF-responsive genes. Moreover, the loss of *Foxi3* can functionally uncouple induction of the otic placode from reception of FGF signaling.

Wnt signaling promotes differentiation of *Pax2*-expressing progenitors into an otic placode fate (Ohyama et al., 2007). In mutants where Wnt signaling is strongly attenuated, these progenitors differentiate into epidermis (Freter et al., 2008; Jayasena et al., 2008; Ohyama et al., 2006). It is therefore possible that the failure to form an otic placode in *Foxi3* mutants might be due to a loss of Wnt signaling. To test whether *Foxi3* mutant ectoderm was still able to respond to Wnt signaling, we examined expression of *Axin2*, a negative regulator of canonical Wnt signaling, which is itself induced by the Wnt pathway (Yamamoto et al., 1998). We observed *Axin2* expression throughout the neural plate and in pre-placodal ectoderm at the level of the presumptive otic placode in

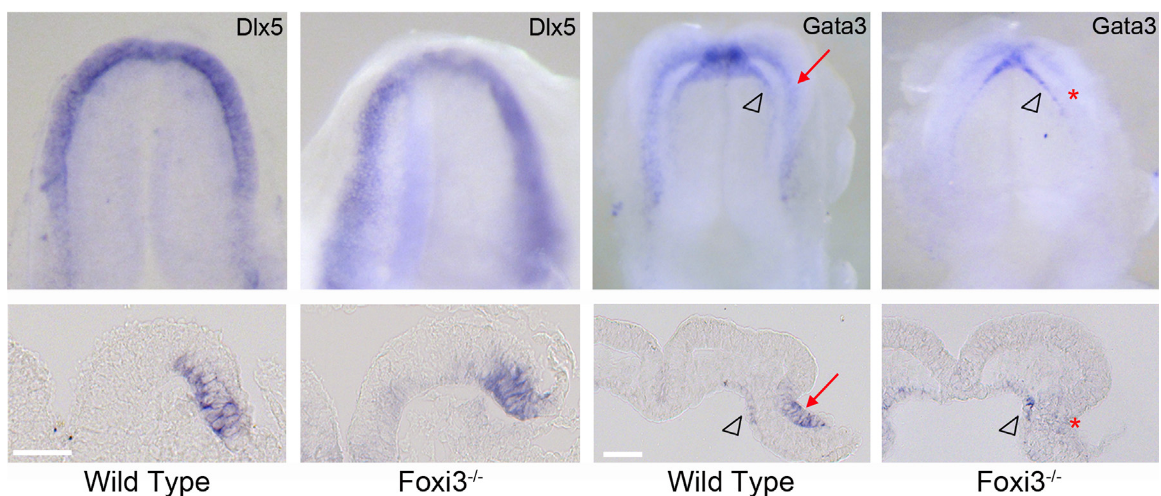

Fig. 5. Expression of non-neural ectoderm transcription factors in head-fold stage *Foxi3* mutants. *Dlx5* expression persists in the non-neural ectoderm of *Foxi3* mutant embryos at the 0-2ss. *Gata3* is expressed in the pre-placodal ectoderm of wild-type embryos (red arrows) but not *Foxi3* mutants (asterisks). However, *Gata3* is still expressed in the anterior endoderm in mutant embryos (open arrowheads). 7–8 wild-type or mutant embryos were examined for each marker. Scale bar = 150 μm.

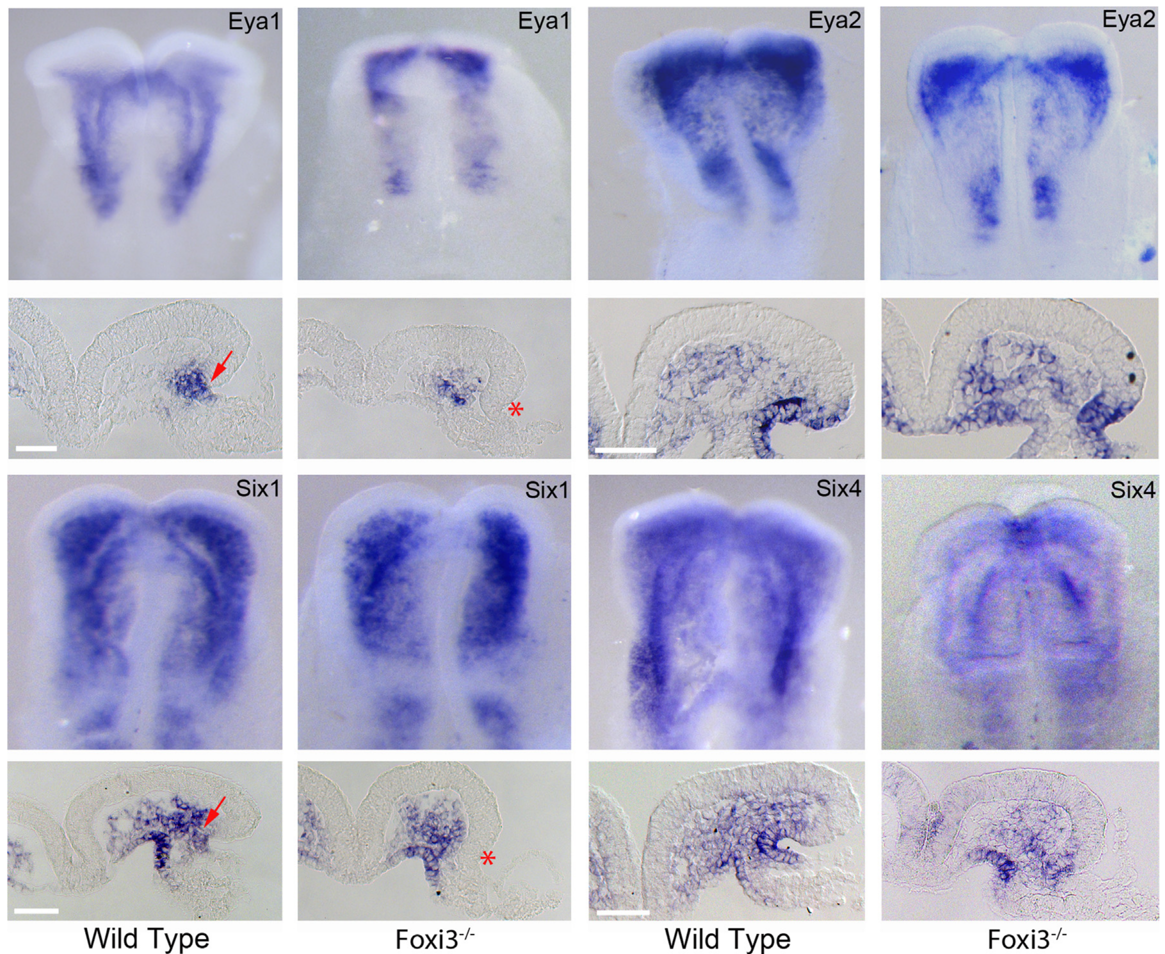

Fig. 6. Differential regulation of the definitive pre-placodal *Six/Eya* genes in *Foxi3* mutants. Analysis of *Six1, Six4, Eya1* and *Eya2* in wild-type and *Foxi3* mutant embryos at the pre-placodal (2-5ss) stage. *Six1* and *Eya1* are absent from the pre-placodal region of *Foxi3* mutants (asterisks) but are expressed in the underlying mesoderm (*Six1, Eya1*) and endoderm (*Eya1) 4* wild-type or mutant embryos were examined for each marker. Pre-placodal expression of *Six1* and *Eya1* in wild-type embryos is indicated with red arrows. In contrast, *Six4* and *Eya2* remain expressed in the pre-placodal ectoderm of *Foxi3* mutants. 6–7 wild-type or mutant embryos were examined for each marker. Scale bar = 150 μm.

wild-type and *Foxi3* mutant embryos (Fig. S4), suggesting that pre-placodal region ectoderm adjacent to the hindbrain still receives and responds to Wnt signaling in *Foxi3* mutants.

3.5. Loss of Foxi3 perturbs the induction of neurogenic but not anterior placodes

Foxi3 is expressed throughout the pre-placodal region at E8.0 and is subsequently down-regulated from the anterior pre-placodal region (Fig. 1). To determine if the transient expression of Foxi3 in the anterior pre-placodal domain was necessary for the induction of anterior placodes, we examined *Foxi3* mutant embryos for expression of Pax2 in the olfactory and lens placodes (Fig. 8). In both cases, Pax2 continued to be expressed in these placodes as previously reported (Ohyama and Groves, 2004b), and we saw no obvious morphological differences in these structures at later stages.

To determine whether posterior neurogenic placodes developed in the absence of Foxi3, we examined *Neurog1* and *Neurog2* expression to observe the development of the trigeminal and epibranchial ganglia, respectively, in E9.5 embryos. *NeuroD* is expressed in all neurogenic ganglia at slightly later stages than *Neurog1* and *2*. *Foxi3* mutant embryos displayed decreased expression of all three markers in the epibranchial and trigeminal ganglia (Fig. 9). Closer examination of *Neurog2* expression showed that the geniculate (G) and petrosal (P) placodes were absent in *Foxi3* mutant embryos. However, *Neurog2* positive cells were still present in the nodose placode (N; Fig. 9). When we visualized the neurons a day later (E10.5) with the Tuj1 anti beta-tubulin antibody, we saw that the seventh, eighth and ninth cranial ganglia were absent from *Foxi3* mutants, as expected since these structures are derived from the missing geniculate, otic and petrosal placodes. However, the nodose ganglion appeared largely unaffected in *Foxi3* mutants.

We also examined the development of the trigeminal ganglion with *Neurog1* and *NeuroD*. In both cases, we observed a smaller patch of cells expressing both markers in *Foxi3* mutants (Tg; Fig. 9). Staining with the TuJ1 antibody at E10.5 showed that the ophthalmic (Vo) and maxillary (Vmx) branches of the trigeminal ganglion were present, but the mandibular branch (Vm) was completely missing in *Foxi3* mutants (Fig. 9). We speculate that the loss of this division of the trigeminal ganglion may be due to the failure of *Foxi3* mutants to develop a first branchial arch (Edlund et al., 2014) into which the mandibular branch of the trigeminal ganglion projects.

Since Foxi2 is expressed extensively in cranial ectoderm, we considered the possibility that Foxi2 could compensate for loss of Foxi3 during nodose and trigeminal ganglia development. *Foxi2* null mutants have no detectable phenotype and breed normally (Ohyama, Edlund and Groves, unpublished observations). We generated *Foxi2;Foxi3* double mutant embryos and compared them to the *Foxi3* single mutant and wild-type embryos. (Fig. 9,

Fig. 7. Fgf signals are presented and received normally in *Foxi3* mutants. The central summary diagram shows a simplified version of the Fgf signaling pathway, with different components examined in panels A–C. (A) *Fgf3* and *Fgf10* are necessary for otic placode induction. Both ligands are expressed normally in *Foxi3* mutant embryos: *Fgf3* is expressed in neural tissue and *Fgf10* is expressed in mesodermal tissue underlying the presumptive otic placode. 3 wild-type or mutant embryos were examined for each marker. (B) *Fgfr2* is expressed in the neural plate and pre-placodal ectoderm (red arrows) of wild-type and *Foxi3* mutant embryos ($n=20$). (C) The *Erm* transcription factor and the Fgf pathway negative regulator *Spry2* are both expressed in *Foxi3* mutant pre-placodal ectoderm (red arrows). 6–9 wild-type or mutant embryos were examined for each marker. Scale bar=300 μm.

right panels) In *Foxi2;Foxi3* double mutants the expression of *Neurog1, Neurog2* and *NeuroD* was not significantly different from *Foxi3* single mutants. Similarly, we saw no difference in TuJ1 staining of the cranial ganglion between *Foxi3* and *Foxi2;Foxi3* mutants at E10.5. This suggests that *Foxi2* and *Foxi3* do not act redundantly in cranial ganglion development.

4. Discussion

A large number of mouse mutants display abnormal development of the inner ear, and many of these mutations manifest their effects at early stages in ear development (Acampora et al., 1999; Bouchard et al., 2010; Burton et al., 2004; Robledo and Lufkin, 2006; Robledo et al., 2002; Torres et al., 1996; Xu et al., 1999;

Fig. 8. The olfactory and lens placodes develop normally in *Foxi3* mutants. Sections through the lens and olfactory regions of E9.5 *Foxi3* mutant and wild-type embryos were stained with antibodies to Pax2 (green) and counter-stained with DAPI (magenta). Both of these anterior placodes develop normally in *Foxi3* mutants. 4 wild-type or mutant embryos were examined.

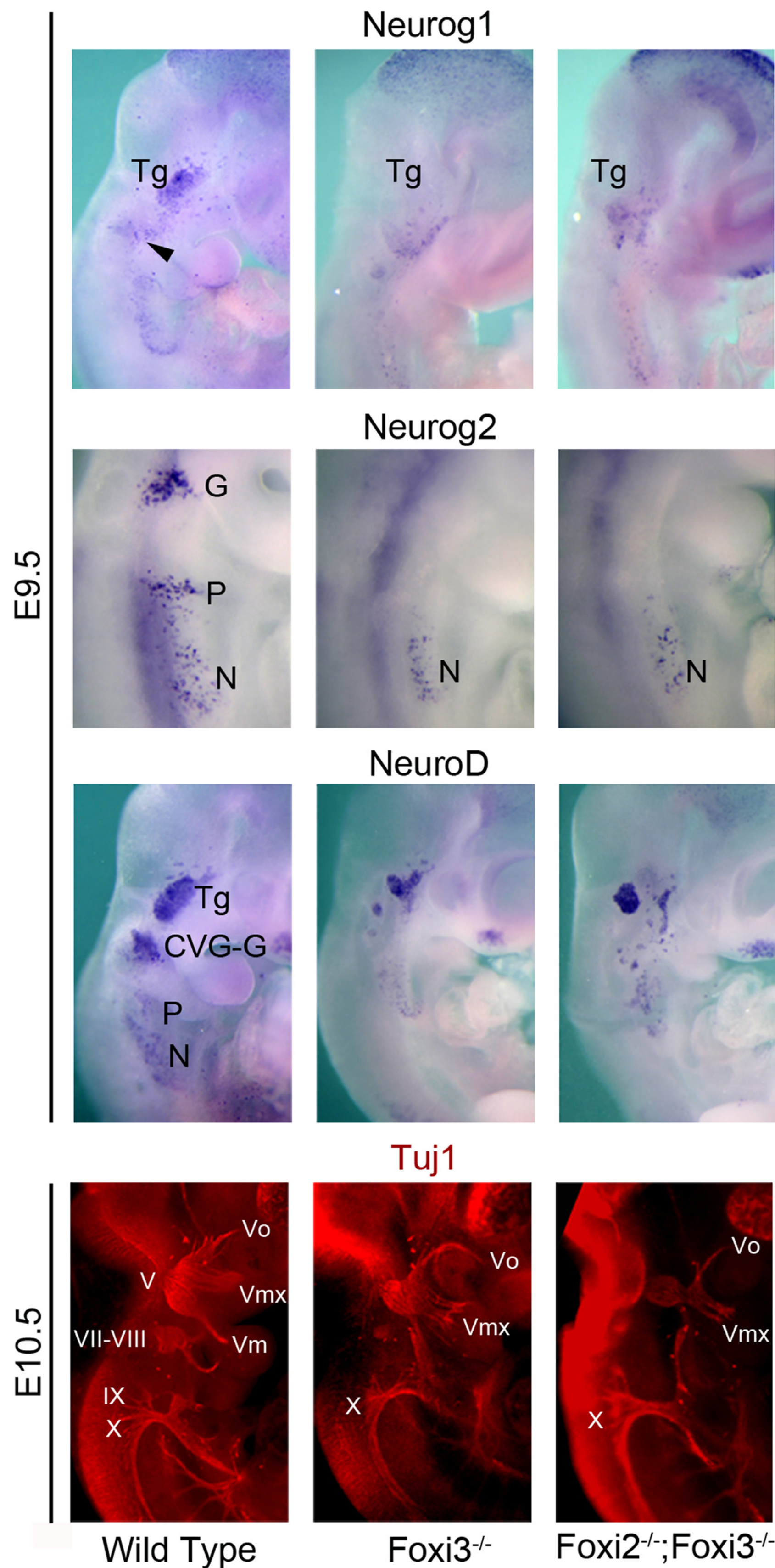

Fig. 9. Loss of *Foxi3* leads to a loss of the geniculate and petrosal placodes and their ganglia and to defects in the mandibular branch of the trigeminal ganglion. E9.5 wild-type, *Foxi3* mutant and *Foxi2;Foxi3* mutant embryos were examined for expression of *Neurog1* and *2* and *NeuroD*. In mutant embryos, *Neurog1* and *NeuroD* expression is reduced in the trigeminal (Vth) ganglion and absent from the cochleovestibular (VIIIth) ganglion (arrowhead in wild type; CVG), consistent with the absence of the otic placode. *Neurog2* is not expressed in the mutant geniculate (G) or petrosal (P) placodes, and *NeuroD* expression in these regions is either absent or strongly reduced. However, the nodose placode (N) expresses *Neurog2* and *NeuroD* at relatively normal levels. 7–8 wild-type, single or double mutant embryos were examined for each marker. At E10.5, Tuj1 staining for neurons shows the absence of the mandibular branch of the trigeminal ganglion (Vm) while the ophthalmic (Vo) and maxillary (Vmx) branches remain. The geniculate and vestibuloacoustic (VII, VIII) ganglia are severely deformed and or absent. The nodose (Xth) ganglion develops normally in all cases. We observed no significant difference between *Foxi3* mutants and *Foxi2;Foxi3* double mutants. 9 wild-type embryos and 4 each of *Foxi3* mutant and *Foxi2;Foxi3* double mutants were examined for TuJ1 staining.

Zheng et al., 2003; Zou et al., 2004). However, to our knowledge, *Foxi3* is the first mouse gene whose loss of function causes a complete failure of the inner ear developmental program from the earliest stages of otic placode induction. *Foxi3* mutant mice fail to express all markers of the otic placode we have tested, including *Sox9*, *Foxg1* and *Dlx5*, as well as *Pax2* and *Pax8,* which are generally accepted as the earliest genes expressed in the otic placode. *Foxi3* mutants also lack any morphological evidence of a thickened or invaginating otic placode. These data suggest that mutation of *Foxi3* completely abolishes the induction of the inner ear.

4.1. Loss of Foxi3 causes a failure of otic placode induction

Foxi3 expression commences at a significantly earlier stage than the onset of otic placode induction. We first observed *Foxi3* expression in the epiblast during gastrulation. Its expression is then refined to non-neural ectoderm and thence to the pre-placodal region before becoming extinguished from the otic placode (Fig. 1). Consequently, there are several possible reasons why otic placode induction does not occur in *Foxi3* mutants. These include the disruption of transcriptional cascades in the non-neural ectoderm or pre-placodal domain, a failure of the pre-placodal ectoderm to receive or respond to FGF signals, and a reduction in survival of otic placode cells as they are induced. Our results suggest that two of these possibilities explain the *Foxi3* mutant phenotype: we see evidence for disrupted gene expression in the pre-placodal domain and some cell death in the otic placode, but the pre-placodal ectoderm itself appears competent to respond to FGF signaling in the absence of *Foxi3*.

The establishment of definitive neural, neural crest, placodal and epidermal territories in the vertebrate head occurs through an iterative process of transcription factor induction and refinement into distinct territories (Grocott et al., 2012; Groves and LaBonne, 2014; Milet and Monsoro-Burq, 2012). This refinement occurs by reciprocal activation of transcription factors in the same territory and mutual repression of transcription factors in adjacent territories. For example, *Dlx5* and *Foxi3* are both initially expressed in non-neural ectoderm before becoming refined to the pre-placodal domain (Fig. S1), and ectopic expression of either factor in chick ectoderm is sufficient to induce expression of the other factor (Khatri et al., 2014). In the present study, we show that *Dlx5* continues to be expressed in *Foxi3* mutants, and although *Foxi3* expression has not been examined in *Dlx5* mutants or *Dlx5;6* double mutants, otic placode induction appears to occur normally in these mutants, with ear defects only apparent at later stages (Acampora et al., 1999; Robledo and Lufkin, 2006). Together, these data suggest that although *Dlx5* and *Foxi3* may reinforce one another's expression, they are not necessary for either the induction or maintenance of each other. We found mouse *Gata3* is expressed at the pre-placodal stage, significantly later than *Foxi3* or *Dlx5,* in contrast with its expression in chicken and zebrafish (Fig. S2). *Gata3* is absent from the pre-placodal region of *Foxi3* mutants (Fig. 5). Morpholino knock down of *foxi1* in zebrafish also reduces *gata3* expression (Bhat et al., 2013) suggesting the regulation of *Gata3* genes by Foxi1/3 factors is conserved between amniotes and anamniotes.

We also observed a loss of *Six1* and *Eya1* specifically from the pre-placodal region of *Foxi3* mutant embryos (Fig. 6). However, *Six4* and *Eya2* are expressed in the pre-placodal region in our mutants. We previously reported that morpholino knockdown of *Foxi3* in stage 3 chick embryos does not affect pre-placodal gene expression (Khatri et al., 2014), suggesting that *Foxi3* acts to promote pre-placodal gene expression at or before this stage in chick. Six1 directly binds to and positively regulates the *Six4* locus in some tissues (Liu et al., 2010), but additional factors likely regulate *Six4* expression in the pre-placodal region. Single mutants of *Six1, Six4, Eya1* and *Eya2* all develop an otic placode and otocyst, although several of these mutants later develop ear defects (Ozaki et al., 2001; Xu et al., 1999; Zheng et al., 2003). *Six1;Six4* and *Eya1; Eya2* double mutants also develop inner ears (Grifone et al., 2007; Zou et al., 2006), as do *Six1;Eya1* mutants, although some studies have reported the otocyst in these mutants to be hypoplastic (Ahmed et al., 2012; Zheng et al., 2003; Zou et al., 2004). Together, these results suggest that the absence of an otic placode in *Foxi3* mutants is likely due to more than the loss of *Six1* and *Eya1* from the pre-placodal region. It is possible that the combined loss of *Six1, Eya1* and *Gata3* in our mutants is sufficient to lead to a failure of otic placode induction, but there are no zebrafish or mouse studies to date in which all three genes have been simultaneously inactivated or knocked down.

What happens to pre-placodal ectoderm that would normally form the otic placode in *Foxi3* mutants? Although this question can only be answered definitively by lineage tracing in *Foxi3* mutant embryos, our data suggest that changes in both cell fate and cell survival may be responsible. We have previously shown that a related Foxi gene, *Foxi2*, is expressed in the epidermis surrounding the otic placode but specifically excluded from the placode itself (Jayasena et al., 2008; Khatri and Groves, 2013; Ohyama and Groves, 2004a; Ohyama et al., 2006). In *Foxi3* mutants, *Foxi2* expression is broader and covers the region where the otic cup would normally form in wild-type embryos. We observed a small but significant increase in apoptotic cells in the pre-placodal region of *Foxi3* mutants. However, the persistence of *Six4*+, *Eya2*+ FGF-responsive ectoderm in *Foxi3* mutants suggests that apoptosis alone cannot account for the absence of the otic placode in *Foxi3* mutants. Rather, it is likely that both a change from otic placode to epidermal fates and compromised survival of presumptive otic progenitors account for the *Foxi3* mutant phenotype.

4.2. Direct and indirect functions of Foxi3 in the induction of other cranial placodes

Foxi3 is initially expressed throughout the developing pre-placodal region before being down-regulated from the anterior neural plate border (Fig. 1). Despite early *Foxi3* expression in the progenitors of the olfactory and lens placodes, we saw no obvious defects in these placodes in *Foxi3* mutants (Fig. 8). This suggests either that *Foxi3* does not regulate anterior placode genes, or that its function can be replaced by other transcription factors in the anterior region, such as *Dlx5* and *Dlx6*, *Pax6* or *Otx2*, all of which are expressed in the anterior pre-placodal region (Bhattacharyya et al., 2004; Khatri et al., 2014; Steventon et al., 2012). In contrast, we observed a reduction in the size of the trigeminal ganglion and an almost complete loss of the petrosal (IXth) and geniculate (VIIth) ganglia (Fig. 9). The mandibular branch of the trigeminal ganglion was the only branch to be significantly compromised in *Foxi3* mutants. Since *Foxi3* mutants have a severely hypoplastic first pharyngeal arch and lack most of the mandible (Edlund et al., 2014), it is possible that the lack of a suitable target is responsible for a failure of this part of the trigeminal ganglion to develop. Likewise, since the epibranchial placodes and their derived ganglia develop in response to signals from the pharyngeal pouches (Begbie et al., 1999; Ladher et al., 2010), the lack of clear pharyngeal pouches in *Foxi3* mutants (Edlund et al., 2014) may result in a failure of epibranchial placode induction. Furthermore, the early loss of *Six1* and *Eya1* from the pre-placodal region of *Foxi3* mutants might be responsible for the loss of the geniculate and petrosal placodes, since these placodes are also absent in *Six1;Eya1* mutant mice (Zou et al., 2004). Interestingly, the nodose (Xth) ganglion is unaffected in both *Foxi3* and *Foxi2;Foxi3* double mutants (Fig. 9). Unlike other epibranchial placodes, the nodose placode arises from a region of ectoderm outside the *Pax2*-expressing otic-

epibranchial precursor domain or OEPD (Freter et al., 2008; Ladher et al., 2010; Ohyama and Groves, 2004b), and so it is possible that either the inductive signals for the nodose placode are distinct from the otic and other epibranchial placodes, or that its competence to respond to placode-inducing signals is regulated by factors distinct from other placodes in the OEPD.

4.3. Amniote Foxi3 and zebrafish foxi1 show sequence and functional homology

The present study and our previous analyses of *Foxi3* in mouse and chick suggest that the amniote *Foxi3* gene is a true homolog of zebrafish *foxi1.* The zebrafish foxi1 protein is more closely related to mouse and chick Foxi3 protein than to either mouse or chick Foxi1 (Edlund et al., 2015). Mouse *Foxi3* mutants completely lack an otocyst and have a greatly reduced lower jaw (Edlund et al., 2014), while zebrafish *foxi1* mutants have a greatly reduced otocyst and jaw, lack expression of many otic placode markers (Hans et al., 2007, 2013; Nissen et al., 2003; Solomon et al., 2003) and have reduced expression of the non-neural ectoderm marker *gata3* (Bhat et al., 2013). Although some preplacodal markers (*six4.1* and *eya1*) are unaffected in *foxi1* morphants (Kwon et al., 2010), no data is available for the expression of these or other preplacodal genes in *foxi1* null mutants. Finally, it should be noted that, just as amniote *Foxi3* and fish *foxi1* appear to be functional homologs, a case can be made for functional homology between amniote *Foxi1* and fish *foxi3.* Here, both genes are expressed later in development and appear to regulate genes involved in the function of transport epithelium. In the case of zebrafish, *foxi3* regulates the development of epidermal ionocytes (Cruz et al., 2013; Esaki et al., 2009; Hsiao et al., 2007; Janicke et al., 2007, 2010; Thermes et al., 2010), while *Foxi1* regulates genes important for production and homeostasis of endolymphatic fluid, such as *SLC26A4* which encodes pendrin (Blomqvist et al., 2004; Hulander et al., 2003; Raft et al., 2014; Vidarsson et al., 2009).

4.4. Is Foxi3 acting as a pioneer factor?

Fgf signaling plays key roles in the early induction of the neural plate border region and its division into the neural plate, neural crest, pre-placodal region and epidermis (Edlund et al., 2015; Grocott et al., 2012; Groves and LaBonne, 2014; Milet and Monsoro-Burq, 2012). Fgf signaling is also necessary for otic placode induction in all vertebrate groups examined to date (Ohyama et al., 2007; Riley and Phillips, 2003; Solomon et al., 2004). Our data suggest that the Fgf signaling pathway remains intact in *Foxi3* mutant mice. The Fgf ligands *Fgf3* and *Fgf10,* together with their receptors *Fgfr1* and *2,* were present in mutant embryos. Furthermore, the expression of the downstream transcription factors *Pea3* (data not shown) and *Erm* was unaffected. Finally, the downstream negative regulator of Fgf signaling, *Spry2* was also expressed in *Foxi3* mutant ectoderm (Fig. 7). In addition, *Axin2* was expressed normally in mutant ectoderm, indicating that the canonical Wnt pathway is activated. Together these data suggest that the action of *Foxi3* is necessary for pre-placodal ectoderm to initiate a program of otic placode induction in response to Fgf and Wnt signals, but is not necessary for the integrity or function of either the Fgf or Wnt pathways. It is therefore possible that *Foxi3* renders pre-placodal cells in a transcriptionally competent state to correctly interpret Fgf and Wnt siganls. Some Forkhead transcription factors are able to access the outer face of DNA in condensed chromatin through their winged helix domains, which have homology to linker histones (Iwafuchi-Doi and Zaret, 2014; Zaret and Carroll, 2011). In this capacity they can act as pioneer factors, recruiting other transcription factors such as Gata family proteins to tissue-specific loci, which become progressively more transcriptionally active (Zaret et al., 2008). Indeed, the zebrafish functional homolog of *Foxi3, foxi1*, remains bound to chromatin during DNA replication (Yan et al., 2006), another hallmark of pioneer factors. At present, we do not know the direct targets of Foxi3 during establishment of the neural plate border region and induction of the otic placode. Moreover, with a few exceptions (Ishihara et al., 2008; Sato et al., 2012), we know very little about the regulatory regions that control expression of pre-placodal and otic placode genes. Identifying these regions and the transcription factors that bind them is a prerequisite for assembling a gene regulatory network for the sensory placodes of the head (Grocott et al., 2012) and for establishing the function of Foxi3 in this regulatory network.

Author summary

Foxi3 is a Forkhead transcription factor that is expressed in the “pre-placodal” domain that gives rise to all vertebrate craniofacial sensory organs, including the inner ear. We generated a mouse knock-out of *Foxi3* and found that the inner ear completely fails to form in these mutants. We saw no molecular evidence for formation of the otic placode, the earliest step in inner ear development. We showed that in the absence of *Foxi3*, three pre-placodal transcription factors-*Gata3*, *Six1* and *Eya1*-are not expressed, indicating that the pre-placodal ectoderm is not properly induced. Fgf signals are essential for embryonic ectoderm to differentiate into the otic placode. We found that Fgf ligands and receptors are correctly expressed in *Foxi3* mutants and that the future ear field receives Fgf signaling. This argues that *Foxi3* mutant cells lack the competence to interpret Fgf signals and are unable to differentiate in response to those signals. Our data suggest that *Foxi3* may be a pioneer factor that enables the cells in the pre-placodal region to interpret the Fgf signal and turn on a program of inner ear induction.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2015.09.022.

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