Developmental Biology 373 (2013) 107–117

Contents lists available at SciVerse ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/locate/developmentalbiology

A gene network that coordinates preplacodal competence and neural crest specification in zebrafish

Neha Bhat, Hye-Joo Kwon, Bruce B. Riley *

Biology Department, Texas A&M University, College Station, TX 77843-3258, United States

ARTICLE INFO

Article history:
Received 15 July 2012
Received in revised form
23 September 2012
Accepted 9 October 2012
Available online 16 October 2012

Keywords:
Zebrafish
Competence factors
Cranial placodes
Neural crest
Heat shock
Transgene
Dorsomorphin

ABSTRACT

Preplacodal ectoderm (PPE) and neural crest (NC) are specified at the interface of neural and nonneural ectoderm and together contribute to the peripheral nervous system in all vertebrates. Bmp activates early steps for both fates during late blastula stage. Low Bmp activates expression of transcription factors Tfap2a and Tfap2c in the lateral neural plate, thereby specifying neural crest fate. Elevated Bmp establishes preplacodal competence throughout the ventral ectoderm by coinducing Tfap2a, Tfap2c, Foxi1 and Gata3. PPE specification occurs later at the end of gastrulation and requires complete attenuation of Bmp, yet expression of PPE competence factors continues well past gastrulation. Here we show that competence factors positively regulate each other's expression during gastrulation, forming a self-sustaining network that operates independently of Bmp. Misexpression of Tfap2a in embryos blocked for Bmp from late blastula stage can restore development of both PPE and NC. However, Tfap2a alone is not sufficient to activate any other competence factors nor does it rescue individual placodes. On the other hand, misexpression of any two competence factors in Bmp-blocked embryos can activate the entire transcription factor network and support the development of NC, PPE and some individual placodes. We also show that while these factors are partially redundant with respect to PPE specification, they later provide non-redundant functions needed for development of specific placodes. Thus, we have identified a gene regulatory network that coordinates development of NC, PPE and individual placodes in zebrafish.

Introduction

Preplacodal ectoderm (PPE) and neural crest (NC) form along neural–nonneural interface near the end of gastrulation and together contribute to the peripheral nervous system. PPE later resolves into a number of sensory placodes that give rise to diverse structures such as the inner ear, olfactory epithelium, lens, anterior pituitary, and parts of trigeminal and epibranchial ganglia (reviewed by Baker and Bronner-Fraser, 2000; Streit, 2007; Schlosser, 2010). Transcription factors encoded by Six, Eya and Dlx gene families are specifically induced or upregulated in the PPE in response to signals from the dorsal tissue, including Fgf, Wnt antagonists and Bmp antagonists (Brugmann et al., 2004; Glavic et al., 2004; Ahrens and Schlosser, 2005; Litsiou et al., 2005; Esterberg and Fritz, 2009; Kwon and Riley, 2009; Kwon et al., 2010). Although PPE formation is restricted to a relatively narrow band abutting the neural plate, the competence or potential to form PPE is present throughout the ventral ectoderm from the beginning of gastrulation (Mancilla and Mayor, 1996; Woda et al., 2003; Glavic et al., 2004; Ahrens and Schlosser, 2005; Kwon et al., 2010). Localized misexpression of Fgf plus Chordin during gastrula stages can induce PPE markers ectopically anywhere within the nonneural ectoderm (Ahrens and Schlosser, 2005; Kwon et al., 2010). We showed previously that competence to form PPE is mediated by a set of four Bmp-activated transcription factor genes, *tfap2a*, *tfap2c*, *foxi1* and *gata3* (Kwon et al., 2010). Knockdown of pairs of competence factors, such as *tfap2a* plus *tfap2c* or *foxi1* plus *gata3*, does not block PPE gene expression, although expression levels are reduced. Knockdown of all four competence factors leads to complete and specific loss of all PPE markers without disrupting the neural plate or epidermal development (Kwon et al., 2010). Thus, these genes are partially redundant and are together necessary for PPE specification.

Although these competence factors are redundant with respect to PPE specification, they appear to play unique roles during later development of various tissues. For example, *foxi1* and *gata3* show dramatic upregulation in the otic/epibranchial placodal region towards the end of gastrulation (Neave et al., 1995; Solomon et al., 2003), due in part to rising levels of Fgf (Padanad et al., 2012). Loss of *foxi1* causes severe deficiency of both otic and epibranchial placodes in zebrafish (Lee et al., 2003; Solomon et al., 2003; Nissen et al., 2003; Sun et al., 2007) whereas loss of *gata3* leads to a severe otic phenotype in mice (Karis et al., 2001).

* Corresponding author. Fax: +1 979 845 2891.
E-mail address: briley@mail.bio.tamu.edu (B.B. Riley).

0012-1606/$ - see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.ydbio.2012.10.012

Likewise, *tfap2a/c* genes have several unique functions: *Tfap2a* has been extensively studied for its essential early role in neural crest development (Luo et al., 2003; Knight et al., 2003; Hoffman et al., 2007; Li and Cornell 2007; Nikitina et al., 2008; Arduini et al., 2009; de Crozé et al., 2011; Wang et al., 2011; Van Otterloo et al., 2012). In zebrafish, disruption of both *tfap2a* and *tfap2c* ablates neural crest and also causes significant deficiencies in all placodal derivatives (Hoffman et al., 2007; Li and Cornell, 2007; Kwon et al., 2010). However, it is not known whether the latter defects reflect disruption of placode formation or faulty regulation of subsequent growth, maintenance and differentiation of placodal derivatives. More generally, the degree to which *tfap2a/c*, *foxi1* and *gata3* can compensate for each other in regulating placodal development remains unexplored.

We showed previously that Bmp is required for the activation but not subsequent maintenance of PPE competence factors at late blastula stage. Expression of competence factors continues at near normal levels even if Bmp signaling is totally blocked from the beginning of gastrulation (Kwon et al., 2010). We speculated then that auto-regulation and cross-regulation amongst competence factors help maintain proper expression levels during PPE specification when Bmp must be attenuated, though this idea has not been tested directly. Similarly, the sufficiency of competence factors to foster PPE development in the complete absence of Bmp remains an open question.

Here we show through gain and loss of function that, despite the partial redundancy of competence factors for PPE specification, competence factors also have unique placode-specific functions. Additionally, during gastrulation these competence factors form a self-sustaining gene regulatory network that maintains proper expression levels even after attenuation of Bmp. In the absence of Bmp, misexpression of *tfap2a* alone cannot activate other competence factors but nevertheless supports both PPE and NC development. Misexpression of any two competence factors in the absence of Bmp signaling not only restores PPE and NC markers but also restores the larger network of competence factors and rescues development of some specific placodes. Thus, we have identified a gene regulatory network that is sufficient, together with appropriate inductive signals, for the formation of sensory organs in zebrafish.

Materials and methods

Developmental conditions

Embryos were incubated in embryo medium (Kimmel et al., 1995) at 28.5 °C, except for heat shock experiments as noted in the text. Wild-type embryos were derived from the AB line.

In situ hybridization and immunostaining

In situ hybridization and immunostaining were performed as previously described (Phillips et al., 2001). Primary antibody for immunostaining against Isl1 was obtained from Hybridoma bank (dilution 1:100) and anti-mouse HRP-conjugated secondary antibody was obtained from Vector labs (dilution 1:200).

Pharmacological treatment

Dorsomorphin (Sigma, P5499) was diluted in DMSO at a concentration of 10 mM. To fully block Bmp signaling, DM stock solution was diluted to a concentration of 200 μM in embryo media. Treatments were conducted in 24-well plates with a maximum of 40 embryos per well in 500 μl of DM solution. Aliquots of DM stock solution were stored frozen at −80 °C indefinitely without detectable loss of activity. Repeated freeze–thaw cycles significantly reduce DM activity. Once thawed, aliquots can be stored for several days at 4 °C. However, long-term storage at 4 °C leads to degradation, with 10% loss of activity after 1 month and 85% loss of activity after 1 year. DM solution should also be shielded from light exposure.

Morpholino injection

For gene knockdown, embryos were injected at the one-cell stage with 5 ng morpholino (1 nl of 5 ng/nl solution), unless otherwise stated. The morpholino sequences used here have been previously tested and published and include *tfap2a* E2I2 splice blocker AGCTTTTCTTCTTACCTGAACATCT (O'Brien et al., 2004); *tfap2c* E3I3 splice blocker TCTGACATCAACTCACCT-GAACATC (Li and Cornell, 2007); *foxi1* translation blocker TAATCCGCTCTCCCTCCAGAAACAT (Solomon et al., 2003); *gata3* translation blocker TCCGGACTTACTTCCATCGTTTATT (Kwon et al., 2010). To assess morphant phenotypes, at least 20 embryos were examined for each experiment unless otherwise stated. For RNA extractions, 100 morphant embryos were used per experiment (see below).

Gene misexpression

Transgenic line *Tg(hsp70:foxi1)*^{x19} was previously described (Kwon et al., 2010). For this study we also generated new lines *Tg(hsp70:tfap2a)*^{x24} and *Tg(hsp70:gata3)*^{x25}: *tfap2a* and *gata3* cDNAs were inserted into *hsp70* vector (Shoji et al., 1998) with I-SceI meganuclease sites (Thermes et al., 2002). 10–40 pg of recombinant plasmid was co-injected with meganuclease (NEB, 0.5 U/μl) into wild-type embryos at the one-cell stage (Rembold et al., 2006). Injected embryos were raised to adulthood and screened for germline transmission by PCR. For simplicity, transgenic lines are referred to in the text as *hs:foxi1*, *hs:tfap2a* and *hs:gata3*, respectively. For misexpression, heterozygous transgenic carriers were heat shocked at 37 °C for 30 min, after which embryos were maintained at 33 °C until fixation. The effects of transgene activation were fully penetrant, except where noted. Phenotypes for double transgenic carriers were readily distinguishable from single transgene carriers. At least 20 embryos were analyzed for each experiment, except for experiments involving RNA extraction for which 100 embryos were used for each experiment (see below).

RNA extraction and cDNA synthesis

For each sample, RNA was extracted from 100 embryos using Trizol, followed by phenol–chloroform extraction. DNA was digested by treating samples with DNAase and RNA was re-extracted by Trizol–phenol–chloroform. cDNA was synthesized from 1 μg of total RNA using an Invitrogen kit. Samples were collected at the end of gastrulation. For misexpression studies, embryos obtained from outcrossing *hs:foxi1*/+, *hs:gata3*/+, and *hs:tfap2a*/+ heterozygotes were heat shocked at 7 hpf at 37 °C for 30 min and RNA was collected at 10 hpf. Corresponding wild-type embryos were also heat shocked to serve as controls.

Real time PCR

For each set of gene-specific primers, cDNA samples were diluted appropriately to generate a linear range of PCR product. For most genes, PCR was performed on template-dilutions of 1:8 and 1:64. For *b-actin* (a constitutive control), template-dilutions were 1:512 and 1:2048. For each independent experiment, two dilutions were repeated in triplicate, and each experiment was repeated three times. 2× Sybr green master mix was prepared by combining the following reagents: Taq buffer, $MgCl_2$, ROX

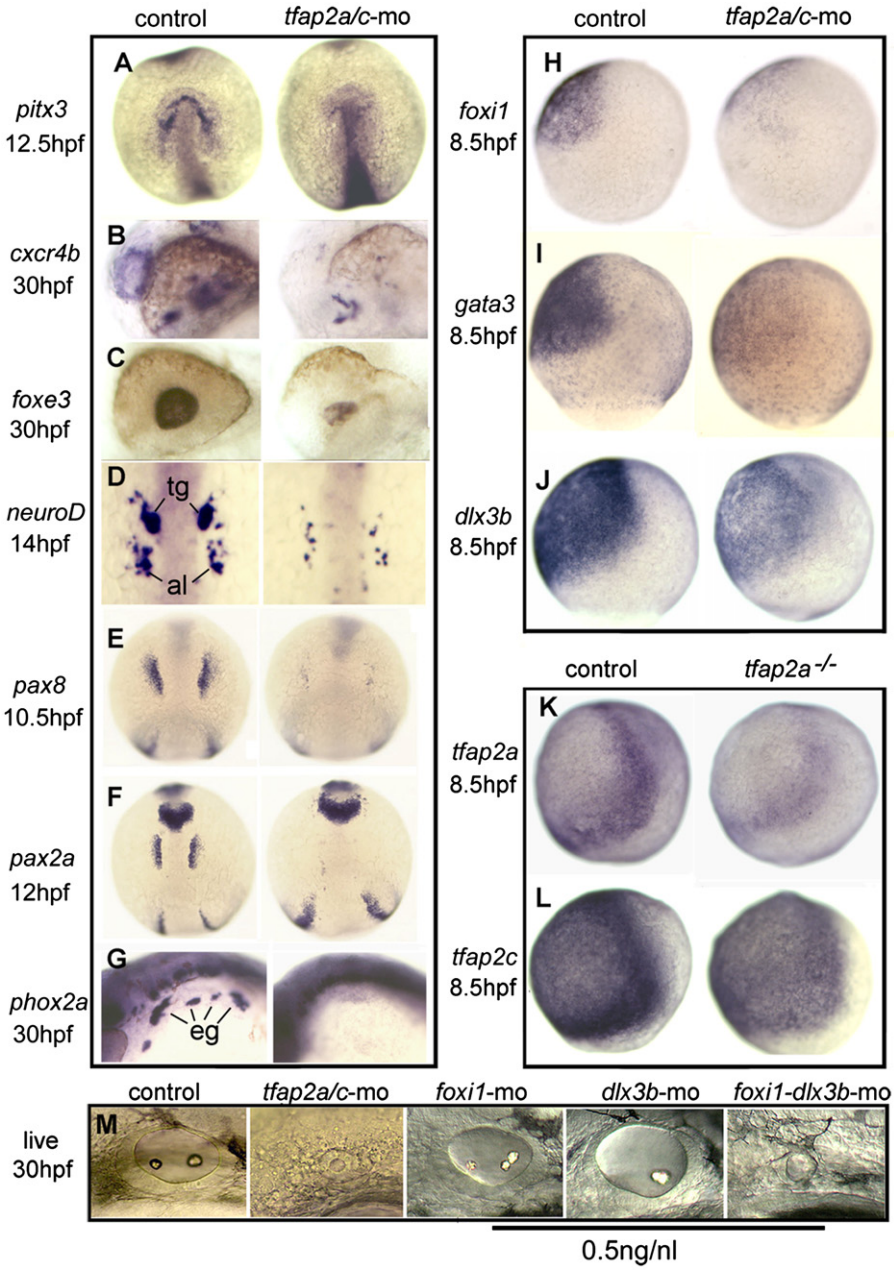

Fig. 1. Knockdown of *tfap2a/c* impairs early placode development. **A–G**, Expression of early placodal markers in control embryos and *tfap2a/c* morphants. Expression of *pitx3* at 12.5 hpf marks pituitary/olfactory/lens precursors (A); *cxcr4b* marks the olfactory pit at 30 hpf (B); *foxe3* marks the lens at 30 hpf (C); *neuroD* marks the trigeminal (tg) and anterior lateral line (al) at 14 hpf (D); *pax8* at 10 hpf (E) and *pax2a* at 12 hpf (F) mark otic/epibranchial precursors; *phox2a* marks epibranchial ganglia (eg) at 30 hpf (G). **H–J**, Expression of competence factors *foxi1* (H) and *gata3* (I), and PPE marker *dlx3b* (J), at 8.5 hpf in control embryos and *tfap2a/c* morphants. **K** and **L**, Expression of *tfap2a* (K) and *tfap2c* (L) at 8.5 hpf in control embryos and *tfap2a*$^{-/-}$ (*lockjaw*) mutants. **M**, otic vesicle at 30 hpf in a control embryo, *tfap2a/c* morphant, and embryos injected with sub-effective doses (0.5 ng) of *foxi1*-MO, *dlx3b*-MO or both *foxi1*-MO and *dlx3b*-MO. Sub-effective doses of these MO have little or no effect by themselves, but when combined have synergistic effects that phenocopy the *tfap2a/c* morphant. Images show dorsal views with anterior up (A, D–F), lateral views with anterior to the left (B, C, G, M) or lateral views with dorsal to the right (H–L).

reference dye, Sybr Green, dNTPs, DEPC H_2O. The 2 × sybr green was mixed with primers and cDNA in the ratio of 5:2:3 in a 10 μl reaction volume. Reactions were performed in 96-well plates (Thermo Scientific, AB-1100) with optical adhesive covers (Applied Biosystems, P/N: 4360954) in an Applied Biosystems 7300 real time PCR system using the default protocol. The fold change was calculated using the $2^{-\Delta\Delta C}{}_T$ method. Extrapolation of transcript levels gave similar values for each dilution, confirming that PCR conditions were held within the linear range. Data from both dilutions from independent experiments were averaged to obtain an overall mean and standard deviation for the sample in question. Data are expressed in fold-change relative to wild-type control levels measured. P-values were calculated using t-tests. For misexpression experiments, only half of embryos were expected to carry the transgene in question, hence the fold change in mRNA level was doubled to compensate.

Results

Requirement for Tfap2a and Tfap2c in formation of cranial placodes

Tfap2a and Tfap2c are best known for their essential roles in neural crest specification (Luo et al., 2003; Knight et al., 2003,

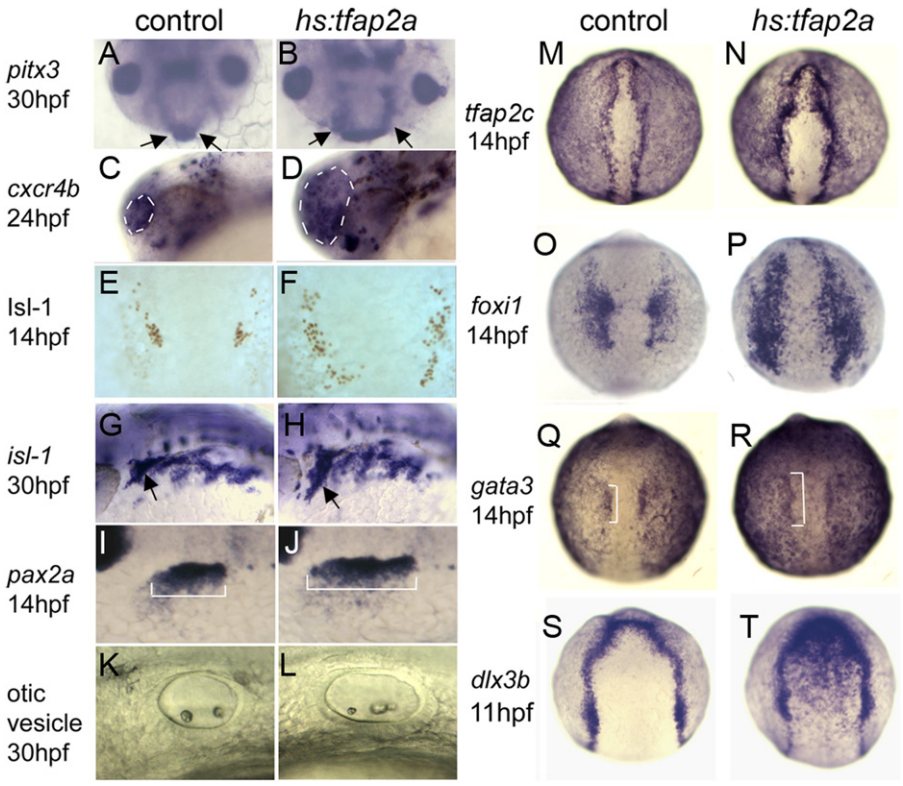

Fig. 2. Misexpression of *tfap2a* promotes overproduction of placodes. **A–L**, Markers of placodal derivatives, including *pitx3* in the anterior pituitary at 30 hpf (A and B, arrows), *cxcr4b* in the olfactory pit at 24 hpf (C and D, circled), Isl1 in trigeminal placode at 14 hpf (E and F), *isl1* in cranial ganglia at 30 hpf (G and H, trigeminal ganglion indicated by arrows), *pax2a* in the otic/epibranchial placodes at 14 hpf (I and J, brackets indicate otic domain), and the otic vesicle at 30 hpf (K and L) in control embryos and *hs:tfap2a*/+ transgenic embryos. All indicated placodal derivatives are enlarged following activation of *hs:tfap2a* at 7 hpf. **M–T**, expression of competence factors *tfap2c* (M and N), *foxi1* (O and P) and *gata3* (Q and R, otic domain indicated by brackets) at 14 hpf, and the general PPE marker *dlx3b* at 11 hpf (S and T) in control embryos and *hs:tfap2a* embryos. Activation of *hs:tfap2a* at 7 hpf results in upregulated and expanded expression of all of these genes in placodal tissues. Images show lateral views with anterior to the left (C, D, G–L), dorsal views with anterior up (E, F, M–T), or facial views of the front of the head (A and B).

2005; Hoffman et al., 2007; Li and Cornell, 2007; Nikitina et al., 2008; de Crozé et al., 2011; Wang et al., 2011). In addition, embryos knocked down for both genes (*tfap2a/c* morphants) show severe deficiencies at 24 hpf in the otic vesicle, olfactory pit and various cranial ganglia (Li and Cornell, 2007), all of which are derived from cranial placodes. However, it was not determined whether defects in placodal derivatives arose from misregulation of initial formation or subsequent growth and maintenance of placodes. By examining early markers, we found *tfap2a/c* morphants are severely deficient in initial formation of all cranial placodes. This includes the anterior pituitary, olfactory and lens placodes (*pitx3*) and subsequent olfactory pit (*cxcr4b*) and developing lens (*foxe3*), the trigeminal placode (*neuroD*), otic placode (*pax8* and *pax2a*), and epibranchial placodes and ganglia (*sox3* and *phox2a*) (Fig. 1A–G, and data not shown). We showed previously that *tfap2a* and *tfap2c* act redundantly with coexpressed genes *foxi1* and *gata3* to establish preplacodal competence in the ventral ectoderm (Kwon et al., 2010). Despite this redundancy, we hypothesized that placodal defects might result from reduced expression levels of competence factors in *tfap2a/c* morphants. In support, knockdown of *tfap2a* and *tfap2c* substantially reduced the level of expression of competence factors *gata3* and *foxi1* (Fig. 1H and I). Expression of *dlx3b*, the earliest known marker of PPE specification, was also reduced in *tfap2a/c* morphants (Fig. 1J). Because the morpholinos for knocking down *tfap2a/c* are splice blockers that destabilize target transcripts, we could not address whether these morpholinos affected feedback regulation of *tfap2a* and *tfap2c* expression. However, *lockjaw* (*low*) mutants, which are disrupted in *tfap2a* function (Knight et al., 2003), were found to express substantially reduced levels of *tfap2a* and *tfap2c* in prospective PPE cells (Fig. 1K and L). Thus, Tfap2a and Tfap2c are required for achieving a proper level of expression of a number of genes acting upstream of PPE specification.

We next tested whether weak impairment of several upstream regulators is sufficient to perturb later placode formation. As a specific example, we tested the effects of injecting sub-effective doses of morpholinos targeting *foxi1* and *dlx3b*, which are critical for proper development of the otic placode (Solomon and Fritz, 2002; Nissen et al., 2003; Liu et al., 2003; Solomon et al., 2003, 2004). Injecting either morpholino alone at 0.5 ng per embryo (10% of the level required to fully knock down gene function) had little effect on early otic gene expression or formation of the otic vesicle (Fig. 1M). However, co-injecting 0.5 ng each of *foxi1*-MO and *dlx3b*-MO caused a severe synergistic deficiency of otic tissue, phenocopying the *tfap2a/c* morphant phenotype (Fig. 1M). Thus, mild impairment of a few upstream regulators is sufficient to explain the deficiencies in otic placode formation seen in *tfap2a/c* morphants. Similar combinatorial reduction seems likely to account for impairment of other placodes as well.

Overexpression of tfap2a expands placodal development

To further investigate the role of *tfap2a* in placodal development, we generated a heat shock inducible transgenic line to misexpress *tfap2a* (*hs:tfap2a*). Full activation of *hs:tfap2a* during gastrulation by heat shocking embryos at 39 °C caused elevated cell death, confounding clear interpretation. However, activation of the transgene at 37 °C did not detectably elevate cell death, so all results reported here are based on heat shocking embryos for 30 min at 37 °C. Activation of *hs:tfap2a* at any time during

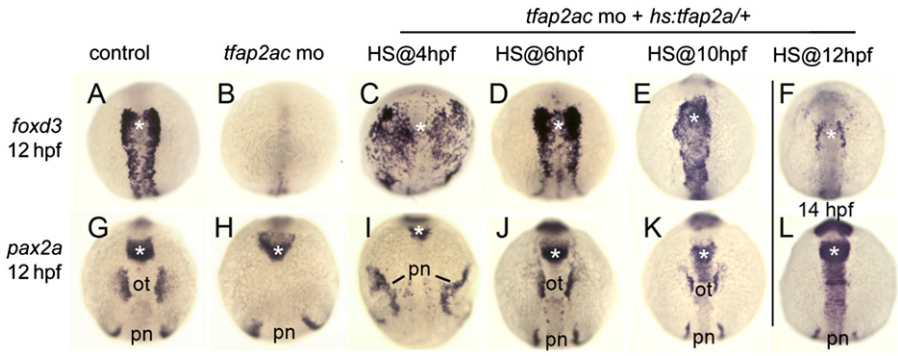

Fig. 3. Temporal requirements for *tfap2a/c*. Expression of neural crest marker *foxd3* at 12 hpf (A–E) and 14 hpf (F), and otic marker *pax2a* at 12 hpf (G-K) and 14 hpf (L) in control embryos (A and G), *tfap2a/c* morphants (B and H), and *tfap2a/c* morphants in which *hs:tfap2a* was activated (HS) at the indicated times (C–F, I–L). Expression domains of *pax2a* in the otic placode (ot) and pronephros (pn) are indicated, and the midbrain–hindbrain border region is marked with an asterisk. Cranial neural crest was rescued by activating *hs:tfap2a* at any time between late blastula stage (4 hpf) and 6 somite-stage (12 hpf), whereas rescue of the otic domain of *pax2a* required activation of the transgene during gastrulation (6–10 hpf). All images show dorsal views with anterior to the top.

gastrulation had similar stimulatory effects on placodal development, though optimal effects as reported here were obtained by heat shocking embryos at 7 hpf. Under these conditions, misexpression of *tfap2a* led to subsequent enlargement of nearly all placodes and their derivatives, including anterior pituitary, olfactory, trigeminal and otic tissues (Fig. 2A–L). Only the lens was relatively unaffected (Fig. 2A, B, and data not shown). Misexpression of *tfap2a* did not detectably increase proliferation based on immunostaining for phosphohistone H3 (not shown). Thus, misexpression of *tfap2a* during gastrulation is sufficient to expand initial domains of most cranial placodes. Activation of *hs:tfap2a* also led to elevated expression of competence factors *tfap2c*, *foxi1*, *gata3* and preplacodal marker *dlx3b* during gastrulation (see below), and significant upregulation of these genes persisted through early- to mid-somitogenesis stages (Fig. 2M–T). Together, these findings suggest that *hs:tfap2a* upregulates expression of upstream regulators of PPE formation, resulting in overproduction of placodal tissue.

Temporal requirement for tfap2a

To establish when *tfap2a* is required for PPE and NC development, we knocked down *tfap2a/c* using splice-blocking morpholinos and then activated *hs:tfap2a* at various times and examined expression of *foxd3* (neural crest) and *pax2a* (otic placode). Note, we focused on *pax2a* because it is the most sensitive indicator for mild impairment of PPE development. As expected, neither marker was expressed in *tfap2a/c* morphants (Fig. 3B and H). Activation of *hs:tfap2a* at late blastula stage (4 hpf) in *tfap2a/c* morphants led to abundant expression of *foxd3* but did not rescue placodal expression of *pax2a* (Fig. 3C and I) or *fgf24* (not shown). In contrast, activation of *hs:tfap2a* in *tfap2ac* morphants at any time during gastrulation (between 6 and 10 hpf) rescued both NC and placodal markers to near wild-type expression patterns (Fig. 3D, E, J and K). Heat shock activation at 12 hpf (6 somites stage) led to weak expression of *foxd3* in cranial neural crest but did not rescue otic expression of *pax2a* (Fig. 3F and L). Thus, the requirement for *tfap2a* by NC can be met at any time between late blastula to early somitogenesis stages whereas the requirement for placodal development occurs during a more restricted interval spanning gastrulation.

The role of foxi1 and gata3 in placodal development

To further investigate unique and redundant functions of PPE competence factors, we examined the effects of knocking down *foxi1*, *gata3* or both *foxi1* and *gata3* (*foxi1-gata3* morphants). Disruption of *foxi1* alone ablates epibranchial placodes and strongly reduces otic placodes (Lee et al., 2003; Nissen et al., 2003; Solomon et al., 2003), and we reported previously that injecting *gata3*-MO alone has no discernable effect on embryonic development (Kwon et al., 2010). In contrast, *foxi1-gata3* morphants show a substantial phenotypic enhancement such that the otic placode is quite small at 13 hpf (Fig. 4D and E) and a morphologically visible otic vesicle fails to form in most specimens (Fig. 4G and H). In addition, the trigeminal placode is reduced by nearly half at 14 hpf (Fig. 4A and B). However, anterior placodes including anterior pituitary (Fig. 4J and K), olfactory pit (Fig. 4M and N) and lens (not shown) develop normally in *foxi1-gata3* morphants, indicating that *foxi1* and *gata3* are specifically required for development of posterior placodes but not anterior placodes.

We next tested whether misexpression of *tfap2a* can compensate for loss of *foxi1* and *gata3*. Anterior placodes were enlarged following activation of *hs:tfap2a* at 7 hpf in *foxi1-gata3* morphants (Fig. 4L and O), but *hs:tfap2a* activation did not improve development of trigeminal, anterior lateral line, otic or epibranchial placodes (Fig. 4C, F and I). Thus, whereas *tfap2a/c* are required for proper development of all cranial placodes, *foxi1* and *gata3* are required only for posterior placodes and their functions cannot be compensated for by elevating *tfap2a*.

To test the effects of misexpressing *foxi1* and *gata3*, we used heat shock inducible transgenic lines and used the same heat shock regimen (37 °C, 30 min) as described above. Activating either *hs:foxi1* or *hs:gata3* alone during gastrulation caused slight enlargement of the otic placode but had no effect on other placodes (not shown). Co-activation of both *hs:foxi1* and *hs:gata3* at 7 hpf caused a more pronounced enlargement of the otic placode (Fig. 5B and F) and the otic vesicle was reproducibly enlarged at 24 hpf (Fig. 5N). Again, no other placodes were affected under these conditions. We next tested whether misexpressing *foxi1* and *gata3* could rescue the placodal defects seen in *tfap2a/c* morphants. Co-activation of *hs:foxi1* and *hs:gata3* restored early markers of the otic and epibranchial placodes to nearly wild-type levels (Fig. 5C, D, G, H, and data not shown) and the otic vesicle also showed substantial recovery (Fig. 5O and P). However, epibranchial ganglia did not form under these conditions, and no other placodes were rescued by *foxi1* and *gata3* (not shown). On the other hand, activation of *hs:foxi1* and *hs;gata3* led to elevated expression of *dlx3b* throughout the preplacodal ectoderm (Fig. 5J) and also restored *dlx3b* levels in *tfap2a/c* morphants (Fig. 5K and L). Together, these data support the conclusion that *foxi1*, *gata3* and *tfap2a/c* genes are partially redundant for PPE specification, but also provide unique (non-redundant) functions during later development of individual placodes.

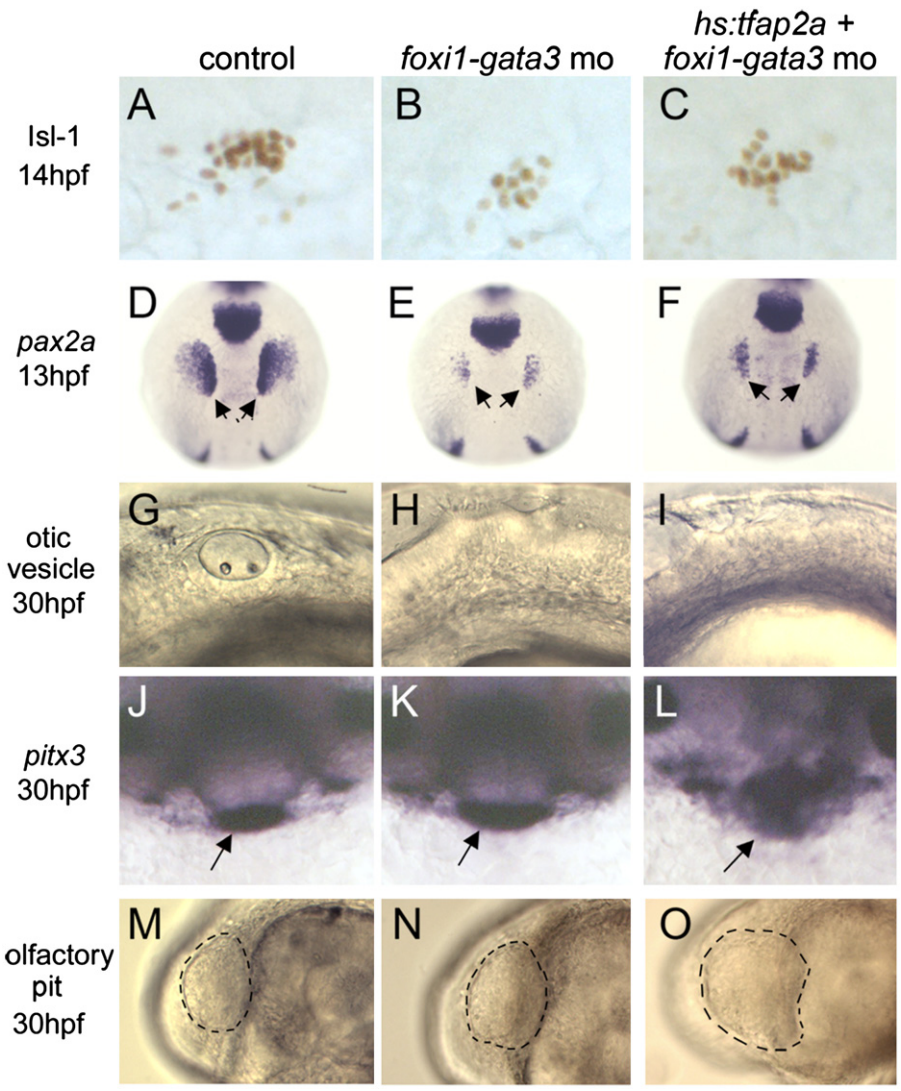

Fig. 4. Unique requirements for *foxi1* and *gata3* in posterior placodes. Markers of cranial placodes and placodal derivatives, including Isl1 in the trigeminal placode at 14 hpf (A–C), *pax2a* in the otic/epibranchial placodes at 13 hpf (D–F, arrows), otic vesicle at 30 hpf (G–I), *pitx3* in the anterior pituitary at 30 hpf (J–L, arrows) and the olfactory pit at 30 hpf (M–O, outlined) in control embryos (A, D, G, J and M), *foxi1-gata3* morphants (B, E, H, K and N), and *foxi1-gata3* morphants in which *hs:tfap2a* was activated at 7 hpf (C, F, I, L and O). Knockdown of *foxi1* and *gata3* reduces the size of the trigeminal, otic and epibranchial placodes, but anterior placodes develop normally. Activation of *hs:tfap2a* does not rescue development of posterior placodes in *foxi1-gata3* morphants, and the transgene still enlarges anterior placodes despite knockdown of *foxi1* and *gata3*. Images show lateral views with anterior to the right (A–C, G–I, M–O), dorsal views with anterior up (D–F), and facial views of the front of the head (J–L).

A self-maintaining genetic network of competence factors

We previously observed that PPE competence factors, once activated by Bmp, no longer require Bmp for their maintenance (Kwon et al., 2010). We hypothesized that competence factors might regulate their own expression during gastrulation, an idea supported by in situ hybridization staining described above. For example, impairment of *tfap2a/c* function appeared to reduce expression levels of all competence factors, as well as PPE marker *dlx3b* (Fig. 1H–L), whereas activation of *hs:tfap2a* appeared to increase expression of these same genes (Figs. 2M–T and 6A, B, D and E). To confirm these results, we measured changes in transcript levels by real time PCR. In support, injection of *tfap2a/c*-MO reduced expression of *foxi1*, *gata3* and *dlx3b* transcript levels by about half (Fig. 6G, H and K, black bars), while activation of *hs:tfap2a* increased expression of these genes by 2- to 3-fold (Fig. 6G, H and K, brown bars). *hs:tfap2a* also doubled the level of *tfap2c* transcript (Fig. 6J, brown bar). Thus, *tfap2a* positively regulates its own expression and expression of other competence factors.

Although *lockjaw* ($tfap2a^{-/-}$) mutants appeared to express reduced levels of *tfap2c* (Fig. 1L), knockdown of *tfap2c* did not signficantly affect *tfap2a* mRNA levels (Fig. 6I, white bar). Thus, despite the general similarity of *tfap2a* and *tfap2c* structure and function, only *tfap2a* plays an essential role in regulating expression levels of both genes (Fig. 6L).

We next measured the effects of manipulating *foxi1* and *gata3*. Injecting *foxi1*-MO caused a 20%–30% decrease in *gata3* and *tfap2a* transcript levels (Fig. 6H and I, gray bars), whereas activation of *hs:foxi1* increased accumulation of *tfap2c* transcript by 70% (Fig. 6J, orange bar). These data suggest that *foxi1* positively regulates these genes. Surprisingly, injecting *gata3*-MO caused a 20%–40% elevation of *tfap2a* and *tfap2c* transcript levels (Fig. 6I and J, green bars). However, co-injection of *gata3*-MO with *foxi1*-MO reduced *tfap2a* and *tfap2c* transcript levels by half (Fig. 6I and J, red bars), reversing the effects of *gata3*-MO alone and strongly enhancing the effects of *foxi1*-MO. Thus, *gata3* function appears highly context-dependent, with *gata3* and *foxi1* together being required to fully activate *tfap2a/c* expression. Activation of *hs:gata3* alone had little effect on any of these genes

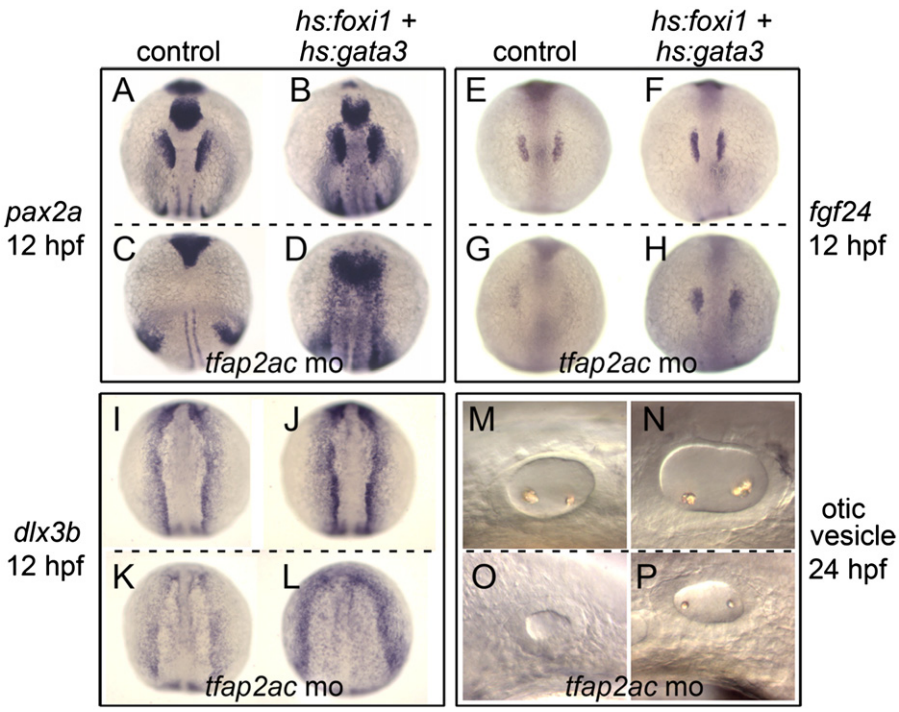

Fig. 5. Co-misexpressing *foxi1* and *gata3* in *tfap2a/c* morphants. **A–D**, Expression of *pax2a* in the otic/epibranchial placodes at 12 hpf. **E–H**, Expression of *fgf24* in the otic placode at 12 hpf. **I–L**, Expression of *dlx3b* in placodal tissues at 12 hpf. **M–P**, otic vesicle at 24 hpf. Non-transgenic controls and *hs:foxi1-hs:*gata3 double transgenic embryos are marked across the top, and the lower half of each panel shows *tfap2a/c* morphants and double transgenic *tfap2a/c* morphants. Activation of the transgenes at 7 hpf partially rescued otic development in *tfap2a/c* morphants. Images show dorsal views with anterior to the top (A–L) or lateral views with anterior to the left (M–P).

(Fig. 6G, I and J, blue bars) despite its ability to enhance the effects of *hs:foxi1* (Fig. 5, and see below).

We next investigated whether *foxi1* and *gata3* regulate their own expression. Injecting *foxi1*-MO increased the level of *foxi1* transcript by 2.5-fold (Fig. 6C and G, gray bar), suggesting an auto-inhibitory loop. Similarly, injecting *gata3*-MO nearly doubled the level of *gata3* transcript (Fig. 6F and H, green bar), suggesting that it, too, is auto-inhibitory. Together, these data support a model in which mutual cross-activation between competence factors, plus auto-activation of *tfap2a*, helps boost expression levels of all competence factors; and the degree of feedback amplification is held in-check by auto-inhibition by *foxi1* and *gata3* (Fig. 6L). Self-regulation of this network likely explains why expression can be maintained even after Bmp is attenuated by signals that specify PPE fate (Kwon et al., 2010). This model is further supported by data provided below.

Sufficiency of competence factors in the absence of Bmp signaling

During late blastula stage, Bmp signaling establishes PPE competence throughout the nonneural ectoderm and, at a lower level, Bmp specifies neural crest along the lateral edges of the neural plate (Nguyen et al., 1998; Tucker et al., 2008; Kwon et al., 2010). Tfap2a and Tfap2c help mediate both of these functions, but it is unknown whether Tfap2a/c is sufficient to mediate all aspects of early Bmp signaling. To test this, we used the pharmacological inhibitor dorsomorphin (DM) (Yu et al., 2008) to fully block Bmp from blastula stage onward and examined whether activating *hs:tfap2a* during gastrulation could rescue later development of PPE and neural crest. Incubation with DM alone (100–200 μM) blocks Bmp signaling immediately and completely, as shown by complete loss phosph-Smad1/5 staining within 15 min (Kwon et al., 2010). Accordingly, treating embryos with 200 μM DM treatment from 4 hpf (late blastula stage) caused complete dorsalization, such that neural plate markers such as *krox20* expanded throughout the DV axis (Fig. 7D) and markers of PPE (*six4.1* and *dlx3b*), neural crest (*foxd3*), and epidermis (*p63*) were lost entirely (Fig. 7A–C, G). As expected, expression of *sizzled*, a direct feedback inhibitor of Bmp signaling (Yabe et al., 2003), was also abolished by DM treatment (Fig. 7H). Activation of *hs:tfap2a* at 6 hpf in DM-treated embryos led to co-expression of *six4.1*, *dlx3b* and *foxd3* by the end of gastrulation (Fig. 7A–C) whereas expression of *p63* was not restored (Fig. 7G). Despite expression of general PPE markers *dlx3b* and *six4.1*, markers of discrete types of placodes (anterior placodes-*pitx3*, otic/epibranchial placodes-*pax8*, trigeminal and anterior lateral line placodes-*neurod*) were not expressed (Table 1). Additionally, activation of *hs:tfap2a* did not activate expression of *foxi1* or *gata3* in DM-treated embryos (Fig. 7E and F, Table 1). Thus, in the absence of Bmp signaling Tfap2a alone is sufficient to provide competence to form PPE and to specify neural crest but cannot activate the larger network of PPE competence factors nor support development of specific placodes.

Interestingly, PPE and neural crest markers induced by activation of *hs:tfap2a* were preferentially expressed on the ventral side of the embryo despite the complete abrogation of Bmp. This likely reflects the action of other factors known to regulate DV patterning. For example, *fgf3* and *fgf8* are still expressed in a DV gradient in DM-treated embryos (not shown), which could influence the ability to express PPE and NC markers. It is also noteworthy that in *hs:tfap2a*-rescued embryos the domains of NC and PPE overlapped in the ventral ectoderm (compare Fig. 7A–C). However, under such conditions cells express one marker or the other, but not both (data not shown, and Kwon et al. (2010)), indicating that cells with distinct fates are intermingled.

We next tested whether other competence factors, alone or in combination, could restore the competence factor network or rescue placodal development in Bmp-blocked embryos. Activation of either *hs:foxi1* or *hs:gata3* in DM-treated embryos led to weak ventrally restricted expression of *tfap2a* and *tfap2c*, as well

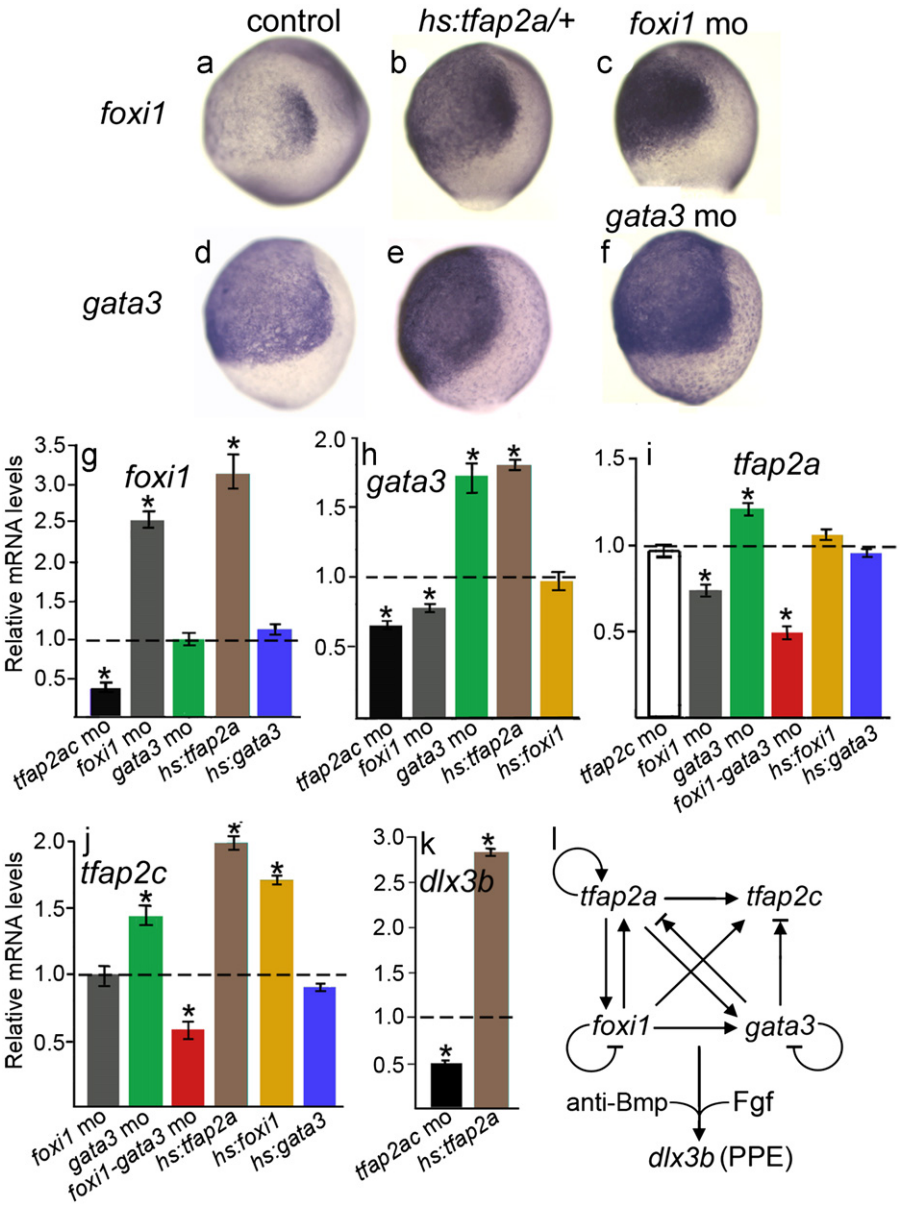

Fig. 6. A network of auto- and cross-regulation amongst competence factors. Changes in expression levels of PPE regulatory genes near the end of gastrulation following misexpression or knockdown of the indicated genes. **A–F**, 10 hpf wholemount expression patterns of *foxi1* (A–C) and *gata3* (D–F) in control embryos (A, D), *hs:tfap2a* transgenic embryos (B and E), a *foxi1* morphant (D) and a *gata3* morphant (F). **G–K**, Quantitative real time PCR measurements of relative mRNA abundance for *foxi1* (G), *gata3* (H), *tfap2a* (I), *tfap2c* (J), and *dlx3b* (K) in the indicated backgrounds. Expression levels were normalized relative to wild-type controls, which are represented by dashed lines set at a value of 1.0. Genetic backgrounds are color coded to facilitate comparison between data sets: Black, *tfap2a/c* morphants; white, *tfap2c* morphants; gray, *foxi1* morphants; green, *gata3* morphants; red, *foxi1-gata3* morphants; brown, *hs:tfap2a*; yellow, *hs:foxi1*; blue, *hs:gata3*. Data represent means of three independent experiments, each performed at two different dilutions, each measured in triplicate. The effects of gene misexpression were assayed at 10 hpf following heat shock at 7 hpf, and corresponding wild-type controls were also heat shocked at 7 hpf. Error bars represent standard deviations. Asterisks indicate statistically significant differences from the controls, as measured by t-tests. **L**, Model and summary of functional relationships between PPE competence factors and the general PPE marker, *dlx3b*. Arrows indicate positive regulation, cross-bars indicate negative regulation, and arrows with cross-bars indicate ambiguity in the data. Specifically, *tfap2a* and *tfap2c* are slightly upregulated in *gata3* morphants but are strongly downregulated in *foxi1-gata3* morphants.

as *foxd3*, but induced little or no expression of PPE or placodal markers (Table 1). Co-activation of *hs:foxi1* and *hs:gata3* had much stronger effects: This led to robust expression of *tfap2a*, *tfap2c*, and *foxd3*, as well as *dlx3b* and *six4.1* (Fig. 8A–D; Table 1) indicating activation of the competence factor network and support of both PPE and neural crest development. Under these conditions, cells expressing *dlx3b* or *foxd3* were observed in largely complementary clusters (Fig. 8E), again suggesting intermingled patterns of cells that adopt either PPE or neural crest fate. In addition to robust expression of general PPE markers, a band of strong *pax8* expression was also induced under these conditions (Fig. 8F), suggesting early stages of otic/epibranchial placode development. Weak expression of *neurod* expression (trigeminal and lateral line placodes) was also seen, although in a very sparse pattern (Fig. 8G, Table 1). Expression of *pitx3* (pituitary, olfactory, lens placodes) was not detected (Table 1).

Next, co-activation of *hs:foxi1* with *hs:tfap2a* led to moderate expression of *tfap2c* and *gata3* in the ventral ectoderm (Table 1), suggesting partial restoration of the competence factor network. Additionally, there was strong but scattered expression of *pax8* and *neurod* (Fig. 8F and G), indicating substantial rescue of posterior placodes. However, expression of *pitx3* (pituitary, olfactory and lens placodes) was not detected. Co-activation of *hs:gata3* with *hs:tfap2a* led to moderate expression of *foxi1*, *tfap2c*

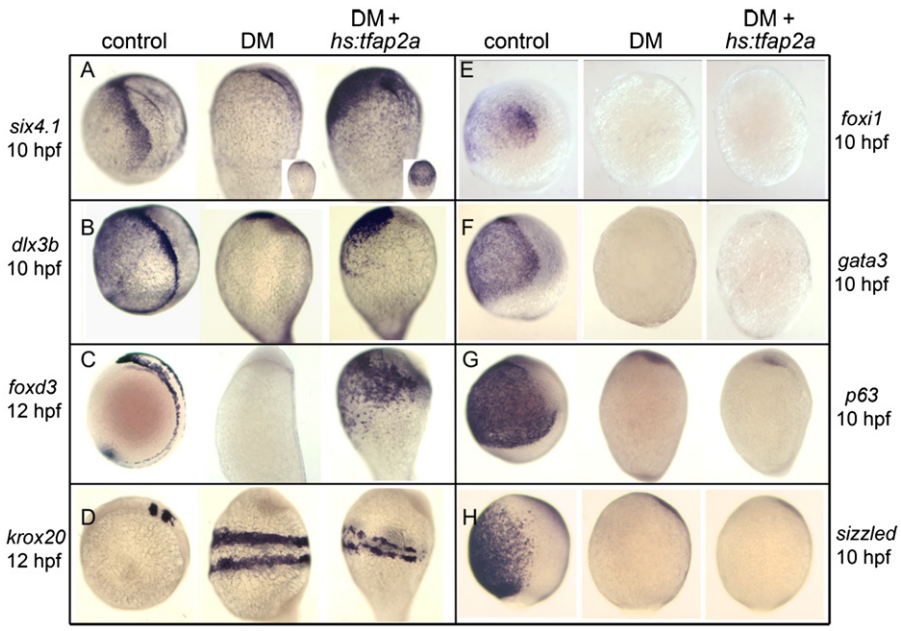

Fig. 7. Misexpression of *tfap2a* rescues PPE and NC in Bmp-blocked embryos. Expression of PPE markers *six4.1* (A) and *dlx3b* (B) at 10 hpf, NC marker *foxd3* at 12 hpf (C), neural plate marker *krox20* at 12 hpf (D), competence factors *foxi1* (E) and *gata3* (F) at 10 hpf, epidermal marker *p63* at 10 hpf (G) and Bmp-feedback inhibitor *sizzled* at 10 hpf (H) in control embryos, embryos treated with 200 μM DM from 4 hpf, or *hs:tfap2a*/+transgenic embryos treated with 200 μM DM from 4 hpf and heat shocked at 6 hpf. Images show lateral views with dorsal to the right and anterior up. Insets in (A) show ventral views of corresponding specimens. Misexpression of *tfap2a* in DM-treated embryos rescues PPE and NC markers while causing slight disruption in stripes of *krox20*, but does not restore expression of other competence factors, *p63* or *sizzled*.

Table 1
Effects of activating transgenes in DM-treated embryos.

Marker gene	Transgenes activated at 6 hpf*					
	hs:foxi1	*hs:gata3*	*hs:tfap2a*	*hs:foxi1+hs:gata3*	*hs:foxi+hs:tfap2a*	*hs:gata3+hs:tfap2a*
foxi1 10 hpf	n.d.	−	−	n.d.	n.d.	+
gata3 10 hpf	−	n.d.	−	n.d.	+	n.d.
tfap2a 10 hpf	±	±	n.d.	++	n.d.	n.d.
tfap2c 10 hpf	±	±	−	+	+	+
foxd3 11 hpf	±	±	+	++	++	++
dlx3b 11 hpf	±	±	+	++	++	++
six4.1 11 hpf	−	−	+	±	++	++
pax8 11 hpf	±	−	−	+	+	±
neurod 14 hpf	−	−	−	±	+	+
pitx3 14 hpf	−	−	−	−	−	−

* Embryos were treated with 200 μM DM beginning at 4 hpf and the indicated transgenes were activated at 6 hpf. Data indicate very strong expression (++), moderate expression (+), weak scattered expression (±), or no expression detected (−). Observed patterns were fully penetrant ($n \geq 15$ embryos for each genotype). n.d., not determined.

and *neurod* (Fig. 8G and Table 1). In this case, however, *pax8* expression was observed in only a few cells in the ventral ectoderm (Fig. 8F). Together, these data indicate that although Bmp is normally required for inducing expression of competence factors, misexpressing any two factors in the absence of Bmp can cross-activate other components of the network and, to varying degrees, restore the expression of markers of specific placodes.

Discussion

We have shown that Tfap2a, Tfap2c, Foxi1 and Gata3 together form a self-maintaining gene regulatory network during gastrulation that fosters competence to form PPE and, in addition, Tfap2a and Tfap2c simultaneously coordinate specification of neural crest along the edges of the neural plate. Robustness of the PPE network arises from mutual cross-activation between its members, and Tfap2a also activates its own expression. The network is also self-limiting because Foxi1 and Gata3 each show auto-repression, thereby preventing unrestrained feedback amplification and assuring maintenance of a proper level of expression in the face of changing signaling interactions that occur during gastrulation. Although competence factors are initially induced by Bmp the network later functions independently of Bmp. Moreover, misexpressing individual competence factors can bypass the need for Bmp. In embryos treated with DM to block all Bmp signaling, the neural plate expands around the entire circumference of the embryo, eliminating NC, PPE and epidermal ectoderm. Activation of *hs:tfap2a* in DM-treated embryos restores both PPE and NC markers but cannot activate the rest of the preplacodal competence network nor support formation of individual placodes. Misexpression of any two PPE competence factors restores the entire competence network and supports formation of NC, PPE and posterior placodes. Epidermal ectoderm is not rescued, confirming that this fate is regulated independently (Kwon et al., 2010). After PPE formation, the full suite of competence factors must be maintained to support subsequent formation of individual placodes. Foxi1 and Gata3 play indispensible roles in development of posterior placodes whereas Tfap2a/c are required

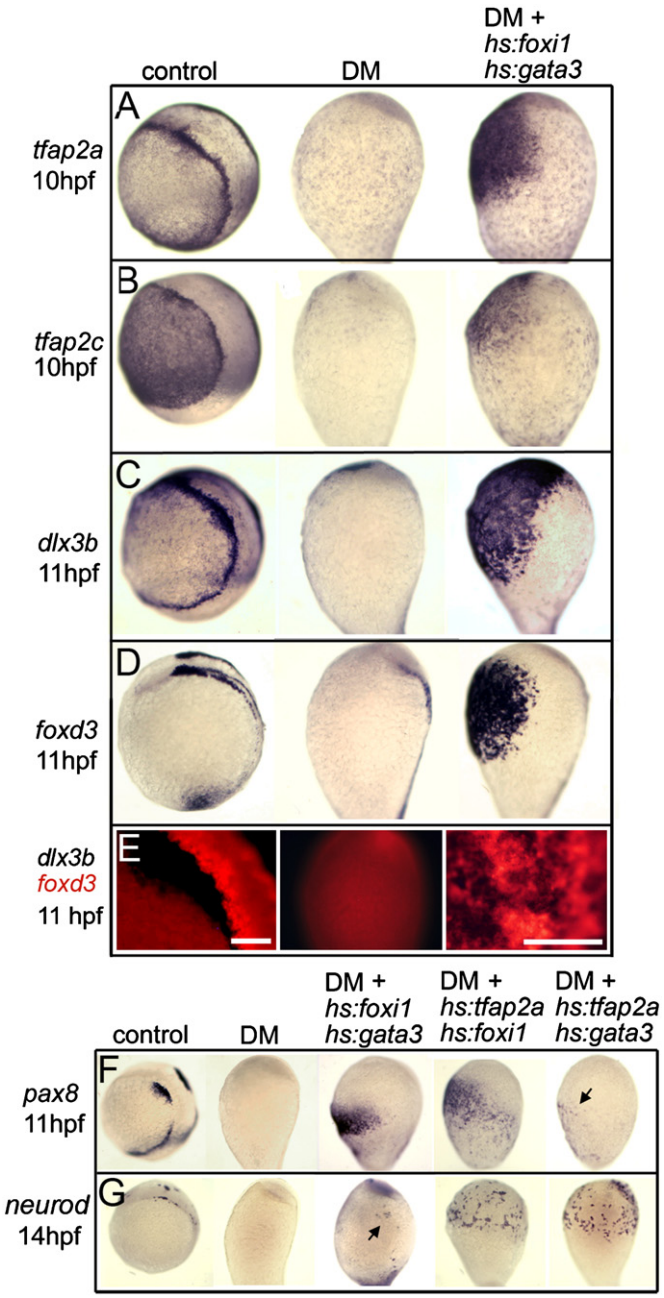

Fig. 8. Effects of co-misexpressing pairs of competence factors in Bmp-blocked embryos. **A–E**, Expression of competence factors *tfap2a* (A) and *tfap2c* (B) at 10 hpf, PPE marker *dlx3b* at 11 hpf (C), NC marker *foxd3* at 11 hpf (D), and two-color in situ hybridization to visualize patterns of *dlx3b* and *foxd3* (E) in controls, embryos treated with 200 μM DM from 4 hpf, and *hs:foxi1/+; hs:gata3/+* transgenic embryos treated with 200 μM DM from 4 hpf and heat shocked at 6 hpf. All markers show rescue in DM-treated embryos following transgene activation. Two-color staining in (E) shows a dorsolateral view of the hindbrain area in a control embryo, a lateral view of a whole DM-treated embryo (dorsal to the right), and a close-up of the ventral midline of a rescued DM-treated transgenic embryo in which *dlx3b* and *foxd3* are expressed in intermingled, largely non-overlapping clumps of cells. Scale bars, 100 μm. **F** and **G**, Expression of otic/epibranchial marker *pax8* at 11 hpf (F) and trigeminal/anterior lateral line marker *neurod* at 14 hpf (G) in control embryos, embryos treated with 200 μM DM from 4 hpf, or in embryos carrying the indicated combinations of transgenes treated with 200 μM DM from 4 hpf and heat shocked at 6 hpf. Arrows point to weak, scattered expression of *pax8* or *neuroD* in various backgrounds.

for achieving sufficient levels of Foxi1, Gata3, and general PPE markers needed for all cranial placodes. These findings extend our previous studies and have important implications for competing models of NC and PPE formation.

Models for coordinating NC and PPE formation

There are currently two competing models for specification of NC vs. PPE. In the "neural border" model, cells lining the neural–nonneural border are initially specified as a distinct zone of common progenitors through early interactions between dorsal and ventral tissues. The NB later subdivides to form NC and PPE in abutting domains depending on differential exposure to Bmp and Wnt (Streit and Stern, 1999; Baker and Bronner-Fraser, 2000; McLarren et al., 2003; Woda et al., 2003; Brugmann et al., 2004; Glavic et al., 2004; Litsiou et al., 2005; Patthey et al., 2008, 2009; de Crozé et al., 2011; Steventon and Mayor, 2012). In the "binary competence" model, neural and nonneural ectoderm are specified early by a gradient of Bmp, with the former harboring neural and NC progenitors and the latter epidermal and PPE progenitors, respectively (Schlosser, 2010; Pieper et al., 2012). That is, PPE and NC potentials are separated quite early, with no intermediate state or stage common to both fates. The data presented here contain aspects consistent with both models, although many of our overall findings are more generally compatible with the binary competence model. Most notably, we reported previously that PPE and NC are specified at different times by different mechanisms (Kwon et al., 2010). NC is specified during late blastula/early gastrula stage by a discrete low level of Bmp (Nguyen et al., 1998; Tucker et al., 2008; Kwon et al., 2010). Conditions that appropriately flatten the Bmp gradient can expand the NC domain to cover the entire ventral half of the ectoderm. In this context, Bmp serves only to induce expression of *tfap2a* and *tfap2c* along the edges of the neural plate; afterwards Bmp is no longer required (Kwon et al., 2010), in sharp contrast to predictions of the neural border model. Unlike NC, PPE is specified later and has two successive steps with opposing Bmp requirements such that no single dose of Bmp can expand PPE fates in a manner similar to NC. Initially, high Bmp levels act in late blastula stage to establish preplacodal competence throughout the non-neural ectoderm, mediated by the overlap of Tfap2a/c, Foxi1 and Gata3 (Kwon et al., 2010). Second, overt specification of PPE occurs near the end of gastrulation and requires stringent attenuation of Bmp by signals from dorsal tissue, including Fgf and Bmp antagonists (Kwon et al., 2010). Misexpression of Fgf8 and Chordin can induce ectopic PPE anywhere within the non-neural ectoderm without co-inducing neural plate or NC (Ahrens and Schlosser, 2005; Kwon et al., 2010). Moreover, when Fgf8 and Chordin are co-misexpressed during late gastrulation, ectopic PPE markers appear quite rapidly thereafter (Kwon et al., 2010), suggesting that ventral ectoderm can develop directly into PPE without first passing through a discrete neural border stage. On the other hand, expression of *tfap2a* and *tfap2c* encompass both PPE and NC domains, providing a mechanistic link between these fates. Accordingly, disruption of *tfap2a/c* ablates neural crest and diminishes, though does not eliminate, PPE (Hoffman et al., 2007; Li and Cornell, 2007; Kwon et al., 2010). This shared early requirement is more consistent with the neural border model. Additionally, misexpression of PPE competence factors in the neural plate (Kwon et al., 2010), or in embryos dorsalized by blocking Bmp (Figs. 7 and 8), result in overlapping domains of PPE and NC. Such patterns reflect intermixing of cells expressing one fate or the other, but not both (Fig. 8E, and Kwon et al., 2010). It is not clear how the two fates are diversified under these conditions since all cells likely experience similar global signals. However, these data do suggest a close kinship between PPE and NC, as predicted by the neural border model. How these data are viewed depends on the relative importance ascribed to initial expression patterns of various regulatory genes, the context in which they act, and whether they specify cell fate directly or merely confer potential.

Acknowledgments

This work was supported by NIH-NIDCD grant R01-DC03806. We thank Matt Sachs and Ying Zhang for technical assistance and use of equipment for conducting real time PCR.

References

Ahrens, K., Schlosser, G., 2005. Tissues and signals involved in the induction of placodal Six1 expression in *Xenopus laevis*. Dev. Biol. 288, 40–59.

Arduini, B.L., Bosse, K.M., Henion, P.D., 2009. Genetic ablation of neural crest diversification. Development 136, 1987–1994.

Baker, C.V.H., Bronner-Fraser, M., 2000. Vertebrate cranial placodes I. Embryonic induction. Dev. Biol. 232, 1–61.

Brugmann, S.A., Pandur, P.D., Kenyon., K.L., Pignoni, F., Moody, S.A., 2004. Six1 promotes a placodal fate within the lateral neurogenic ectoderm by functioning as both a trancriptional activator and repressor. Development 131, 5871–5881.

de Crozé, N., Maczkowaiak, R., Monsoro-Burq, H., 2011. Reiterative AP2a activity controls sequential steps in the neural crest gene regulatory network. Proc. Natl. Acad. Sci. USA 108, 155–160.

Esterberg, R., Fritz, A., 2009. dlx3b/4b are required for the formation of preplacodal region and otic placode through modulation of Bmp activity. Dev. Biol. 325, 189–199.

Glavic, A., Maris Honore, S., Gloria Feijoo, C., Bastidas, F., Allende, M.L., Mayor, R., 2004. Role of BMP signaling and the homeoprotein Iroquois in the specification of the cranial placodal field. Dev. Biol. 272 (1), 89–103.

Hoffman, T.L., Javier, A.L., Campeau, S.A., Knight, R.D., Schilling, T.F., 2007. Tfap2 transcription factors in zebrafish neural crest development and ectodermal evolution. J. Exp. Zool. 308B, 679–691.

Kimmel, C.B., Ballard, W.W., Kimmel, S.R., Ullmann, B., Schilling, T.F., 1995. Stages of embryonic development of the zebrafish. Dev. Dyn. 203, 253–310.

Karis, A., Pata, I., van Doorninck, J.H., Grosveld, F., de Zeeuw, C.I., de Caprona, D., Fritzsch, B., 2001. Transcription factor GATA-3 alters pathway selection of olivocochlear neurons and affects morphogenesis of the ear. J. Comp. Neurol. 429, 615–630.

Knight, R.D., Javidan, Y., Zhang, T., Nelson, S., Schilling, T.F., 2005. AP2-dependent signals from ectoderm regulate craniofacial development in the zebrafish. Development 132, 3127–3138.

Knight, R.D., Nair, S., Nelson, S.S., Afshar, A., Javidan, Y., Geisler, R., Rauch, G.J., Schilling, T.F., 2003. Lockjaw encodes a zebrafish tfap2a required for early neural crest development. Development 130, 5755–5768.

Kwon, H.J., Riley, B.B., 2009. Mesendodermal signals required for otic induction: Bmp-antagonists cooperate with Fgf and can facilitate formation of ectopic otic tissue. Dev. Dyn. 238, 1582–1594.

Kwon, H.J., Bhat, N., Sweet, E.M., Cornell, R.A., Riley, B.B., 2010. Identification of early requirements for preplacodal ectoderm and sensory organ development. PLoS Genet. 6 (9), e1001133.

Lee, S.A., Shen, E.L., Fiser, A., Sali, A., Guo, S., 2003. The zebrafish forkhead transcription factor Foxi1 specifies epibranchial placode-derived sensory neurons. Development 130, 2669–2679.

Li, W., Cornell, R.A., 2007. Redundant activities of Tfap2a and Tfap2c are required for neural crest induction and development of other non-neural ectoderm derivatives in zebrafish embryos. Dev. Biol. 304, 338–354.

Litsiou, A., Hanson, S., Streit, A., 2005. A balance of FGF, BMP and WNT signalling positions the future placode territory in the head. Development 132, 4051–4062.

Liu, D., Chu, H., Maves, L., Yan, Y.L., Morcos, P.A., Postlethwait, J.H., Westerfield, M., 2003. Fgf3 and Fgf8 dependent and independent transcription factors are required for otic placode specification. Development 130, 213–2224.

Luo, T., Lee, Y.H., Saint-Jeannet, J.P., Sargent, T.D., 2003. Induction of neural crest in *Xenopus* by transcription factor AP2α. Proc. Natl. Acad. Sci. USA 100, 532–537.

Mancilla, A., Mayor, R., 1996. Neural crest formation in *Xenopus laevis*: mechanisms of Xslug induction. Dev. Biol. 177, 580–589.

McLarren, K.W., Litsiou, A., Streit, A., 2003. DLX5 positions the neural crest and preplacode region at the border of the neural plate. Dev. Biol. 259, 34–47.

Neave, B., Rodaway, A., Wilson, S.W., Patient, R., Holder, N., 1995. Expression of zebrafish GATA 3 (*gata3*) during gastrulation and neurulation suggests a role in the specification of cell fate. Mech. Dev. 51, 169–182.

Nguyen, V.H., Schmid, B., Trout, J., Connors, S.A., Ekker, M., Mullins, M.C., 1998. Ventral and lateral regions of the zebrafish gastrula, including the neural crest progenitors, are established by a bmp2b/swirl pathway of genes. Dev. Biol. 199, 93–110.

Nikitina, N., Sauka-Spengler, T., Bronner-Fraser, M., 2008. Dissecting early regulatory relationships in the lamprey neural crest gene network. Proc. Natl. Acad. Sci. USA 105, 20083–20088.

Nissen, R.M., Yan, J., Amsterdam, A., Hopkins, N., Burgess, S.M., 2003. Zebrafish foxi one modulates cellular responses to Fgf signaling required for the integrity of ear and jaw patterning. Development 130, 2543–2554.

O'Brien, E.O., d'Alencon, C., Bonde, G., Li, W., Schoenebeck, J., Allende, M.L., Gelb, B.D., Yelon, D., Eisen, J., Cornell, R.A., 2004. Transcription factor Ap-2α is necessary for development of embryonic melanophores, autonomic neurons and pharyngeal skeleton in zebrafish. Dev. Biol. 265, 246–261.

Padanad, M.S., Bhat, N., Guo, B., Riley, B.B., 2012. Conditions that influence the response to Fgf during otic placode induction. Dev. Biol. 364, 1–10.

Patthey, C., Gunhaga, L., Edlund, T., 2008. Early development of the central and peripheral nervous systems is coordinated by Wnt and Bmp signals. PLoS One 3, 31625.

Patthey, C., Gunhaga, L., Edlund, T., 2009. Wnt-regulated temporal control of BMP exposure directs the choice between neural plate border and epidermal fate. Development 136, 73–83.

Phillips, B.T., Bolding, K., Riley, B.B., 2001. Zebrafish *fgf3* and *fgf8* encode redundant functions required for otic placode induction. Dev. Biol. 235, 351–365.

Pieper, M., Ahrens, K., Rink, E., Peter, A., Schlosser, G., 2012. Differential distribution of competence for panplacodal and neural crest induction to non-neural and neural ectoderm. Development 139, 1175–1187.

Rembold, M., Lahiri, K., Foulkes, N.S., Wittbrodt, J., 2006. Transgenesis in fish: efficient selection of transgenic fish by co-injection with a fluorescent reporter construct. Nat. Protoc. 1, 1133–1139.

Schlosser, G., 2010. Making senses: development of vertebrate cranial placodes. Int. Rev. Cell Mol. Biol. 283, 129–234.

Shoji, W., Yee, C.S., Kuwada, J.Y., 1998. Zebrafish Semaphorin Z1a collapses specific growth cones and alters their pathway in vivo. Development 125, 1275–1283.

Solomon, K.S., Fritz, A., 2002. Concerted action of two *dlx* paralogs in sensory placode formation. Development 129, 3127–3136.

Solomon, K.S., Kwak, S.J., Fritz, A., 2004. Genetic interactions underlying otic placode induction and formation. Dev. Dyn. 230, 419–433.

Solomon, K.S., Kudoh, T., Dawid, I.B., Fritz, A., 2003. Zebrafish *foxi1* mediates otic placode formation and jaw development. Development 130, 929–940.

Steventon, B., Mayor, R., 2012. Early neural crest induction requires an initial inhibition of Wnt signals. Dev. Biol. 365, 196–207.

Streit, A., 2007. The preplacodal region: an ectodermal domain with multipotential progenitors that contribute to sense organs and cranial sensory ganglia. Int. J. Dev. Biol. 51 (6–7), 447–461.

Streit, A., Stern, C.D., 1999. Establishment and maintenance of the border of the neural plate in the chick: involvement of FGF and BMP. Mech. Dev. 82, 51–66.

Sun, S.K., Dee, C.T., Tripathi, V.B., Rengifo, A., Hirst, C.S., Scotting, P.J., 2007. Epibranchial and otic placodes are induced by a common Fgf signal, but their subsequent development is independent. Dev. Biol. 303 (2), 675–686.

Thermes, V., Grabher, C., Ristoratore, F., Bourrat, F., Choulika, A., Wittbrodt, J., Joly, J.S., 2002. *I-SceI* meganuclease mediates highly efficient transgenesis in fish. Mech. Dev. 118, 91–98.

Tucker, J.A., Mintzer, K.A., Mullins, M.C., 2008. The BMP signaling gradient patterns dorsoventral tissues in a temporally progressive manner along the anteroposterior axis. Dev. Cell 14, 108–119.

Van Otterloo, E., Li, W., Garnett, A., Cattell, M., Meulemans Medeiros, C., Cornell, R.A., 2012. Novel Tfap2-mediated control of *soxE* expression facilitated the evolutionary emergence of the neural crest. Development 139, 720–730.

Wang, W.D., Melville, D.B., Monteror-Balaguer, M., Hatzopoulos, A.K., Knapik, E.W., 2011. Tfap2a and Foxd3 regulate early steps in the development of the neural crest progenitor population. Dev. Biol. 360, 173–185.

Woda, J.M., Pastagia, J., Mercola, M., Artinger, K.B., 2003. Dlx proteins position the neural plate border and determine adjacent cell fates. Development 130, 331–342.

Yabe, T., Shimizu, T., Muraoka, O., Bae, Y.K., Hirata, T., Nojima, H., Kawakami, A., Hirano, T., Hibi, M., 2003. Ogon/secreted frizzled functions as a negative feedback regulator of Bmp signaling. Development 130, 2705–2716.

Yu, P.B., Hong, C.C., Sachidanandan, C., Babitt, J.L., Deng, D.Y., Hoyng, S.A., Lin, H.Y., Bloch, K.D., Peterson, R., 2008. Dorsomorphin inhibits Bmp signals required for embryogenesis and iron metabolism. Nat. Chem. Biol. 4, 33–41.