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Differential distribution of competence for panplacodal and neural crest induction to non-neural and neural ectoderm

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SUMMARY

It is still controversial whether cranial placodes and neural crest cells arise from a common precursor at the neural plate border or whether placodes arise from non-neural ectoderm and neural crest from neural ectoderm. Using tissue grafting in embryos of *Xenopus laevis*, we show here that the competence for induction of neural plate, neural plate border and neural crest markers is confined to neural ectoderm, whereas competence for induction of panplacodal markers is confined to non-neural ectoderm. This differential distribution of competence is established during gastrulation paralleling the dorsal restriction of neural competence. We further show that Dlx3 and GATA2 are required cell-autonomously for panplacodal and epidermal marker expression in the non-neural ectoderm, while ectopic expression of Dlx3 or GATA2 in the neural plate suppresses neural plate, border and crest markers. Overexpression of Dlx3 (but not GATA2) in the neural plate is sufficient to induce different non-neural markers in a signaling-dependent manner, with epidermal markers being induced in the presence, and panplacodal markers in the absence, of BMP signaling. Taken together, these findings demonstrate a non-neural versus neural origin of placodes and neural crest, respectively, strongly implicate Dlx3 in the regulation of non-neural competence, and show that GATA2 contributes to non-neural competence but is not sufficient to promote it ectopically.

KEY WORDS: Cranial placodes, *Xenopus*, Dlx3, GATA2

INTRODUCTION

Neural crest and cranial placodes are specialized ectodermal tissues that give rise to many different cell types. Whereas the neural crest forms sensory neurons, glial cells, pigmented cells, and cranial bone and cartilage, placodes form sensory neurons and contribute to many cranial sense organs. It is long known that the neural crest originates at the neural plate border (Morales et al., 2005; Kuriyama and Mayor, 2008; Sauka-Spengler and Bronner-Fraser, 2008). Recently, it has become evident that all placodes arise from an adjacent territory surrounding the anterior neural plate, the so-called pre-placodal ectoderm, which expresses the panplacodal markers *Six1* and *Eya1* (Streit, 2007; Schlosser, 2010). Based on this proximity in position, shared gene expression patterns and similarities in cell fate, it has been suggested that neural crest and placodes develop from a common neural plate border region, initially specified by neural plate border specifier genes (Streit and Stern, 1999; Baker and Bronner-Fraser, 2001; McLarren et al., 2003; Woda et al., 2003; Glavic et al., 2004; Meulemans and Bronner-Fraser, 2004; Brugmann et al., 2004; Litsiou et al., 2005; Patthey et al., 2008; Patthey et al., 2009). Subsequently, specific signals induce neural crest specifier genes medially and panplacodal genes laterally within the border.

In favor of this model, previous experiments suggested that grafting neural plates into epidermis induces neural crest and the panplacodal marker *Six1* on both sides of the graft boundary (Moury and Jacobson, 1990; Selleck and Bronner-Fraser, 1995; Mancilla and Mayor, 1996; Litsiou et al., 2005). However, these experiments were not entirely conclusive (Schlosser, 2006) and other studies found neural crest only to be induced from neural plate, whereas panplacodal markers were only induced in the epidermis (Liem et al., 1995; Dickinson et al., 1995; Basch et al., 2000; Glavic et al., 2004; Ahrens and Schlosser, 2005). We, therefore proposed an alternative binary competence model, according to which only neural ectoderm is competent to form neural crest, while only non-neural ectoderm is competent to develop into placodal fates (Ahrens and Schlosser, 2005; Schlosser, 2006).

We here conduct extensive grafting experiments in *Xenopus laevis* to show that at neural plate stages competence for induction of neural plate, border and crest markers is indeed restricted to neural ectoderm, whereas competence for induction of panplacodal markers is confined to non-neural ectoderm in agreement with the binary competence model. These differences in competence are established during gastrulation paralleling the dorsal restriction of neural competence.

The early confinement of *Dlx3*, *Dlx5*, *GATA2* and *GATA3* expression to non-neural ectoderm (Dirksen et al., 1994; Walmsley et al., 1994; Kelley et al., 1994; Read et al., 1998; Feledy et al., 1999; Luo et al., 2001a; Luo et al., 2001b; Schlosser and Ahrens, 2004) suggests that these genes may control non-neural competence. Indeed, Dlx3/Dlx5 and GATA2/GATA3 have been implicated in positioning the neural plate border and are required for induction of panplacodal markers (Pera et al., 1999; McLarren et al., 2003; Woda et al., 2003; Linker et al., 2009; Esterberg and Fritz, 2009; Kwon et al., 2010). However, Dlx3/Dlx5 are also required for induction of neural crest and Rohon Beard neurons, while the effects of GATA factors on neural crest development have not been studied (McLarren et al., 2003; Woda et al., 2003; Kaji and Artinger, 2004; Rossi et al., 2009). Moreover, Dlx3/Dlx5 overexpression in neural ectoderm was insufficient to upregulate

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epidermal and most panplacodal markers (McLarren et al., 2003; Woda et al., 2003). Therefore, the precise role of these transcription factors at the neural plate border is currently unclear.

Using gain- and loss-of-function approaches, we here clarify the role of Dlx3 and GATA2 and show that both are required cell-autonomously for panplacodal and epidermal marker expression in non-neural ectoderm. Ectopic expression of Dlx3 (but not GATA2) in the neural plate is sufficient to induce non-neural markers in a signaling-dependent manner, whereas neural plate, border and crest markers are suppressed after Dlx3 or GATA2 overexpression. These findings confirm the binary competence model, strongly implicate Dlx3 in the regulation of non-neural competence, and show that GATA2 contributes to non-neural competence but is not sufficient to promote it ectopically.

MATERIALS AND METHODS

Expression constructs

The following constructs were used to obtain mRNAs: *Dlx3*, pCS2$^+$EBMyc-Dlx3 (kindly provided by Dr T. Sargent, NICHD, Rockville, MD, USA) consists of the *Eco*RI-*Bam*HI fragment of pCTS-Dlx3 (Feledy et al., 1999) subcloned into pCS2$^+$EBMyc (pCS2+ with the *Bam*HI-*Xba*I part of the polylinker replaced with oligo 5′-GATCTGGC ATG GAG CAA AAG CTC ATT TCT GAA GAG GAC TTG AAT GAA TTC ATC GAT GGA TCC TAG G-3′, containing *Eco*RI-*Cla*I-*Bam*HI sites, a single Myc tag at the 5′ end and a stop codon at the 3′ end); *Dlx5*, pCTS-Dlx5 (Luo et al., 2001b); *EnR-Dlx3hd*, pCS2$^+$EnR-Dlx3hd (Woda et al., 2003); *GATA2*, pSP64T-GATA2a (Weber et al., 2000); *GATA3*, *Xenopus GATA3* (accession number BC110754) cloned into pExpress-1 (ImaGenes); *GATA2znf-EnR*, pG2En (Sykes et al., 1998); and *FGF8*, pCS2+XFGF8 (Christen and Slack, 1997).

Morpholinos

Translation blocking morpholino antisense oligonucleotides (MO) were generated against *Dlx3* and *GATA2* (GeneTools). The Dlx3 MO (5′-CAGAGCCGGAGAAACGAACCAGACT-3′) targeted base pairs –32 to –8 of the *Dlx3* 5′UTR, whereas the GATA2 MO (5′-GGCCACTTCCATCGCAGGAGCAAAG-3′) targeted base pairs –13 to 12 spanning the start of the *GATA2* coding sequence. A standard MO (5′-CCTCTTACCTCAGTTACAATTTATA-3′; GeneTools) was used as a control. Efficacy and specificity of Dlx3 MO was verified in western blots following in vitro transcription and translation (TNT-coupled reticulocyte lysate kit, Promega) of full-length *Dlx3* (BC123268) or *Dlx5* (BC074492), cloned into pExpress-1 (ImaGenes) with and without Dlx3 MO. Equal loading was verified by Coomassie staining. Efficacy and specificity of GATA2 MO was similarly verified using full-length *GATA2* (BC108544) or *GATA3* (BC110754) cloned into pExpress-1 (ImaGenes) with and without GATA2 MO. Plasmid (1 μg), 8.5 μg MO and 1 μl biotinylated lysine tRNA (Transcend, Promega) were used per 25 μl TNT reaction. Subsequently, biotinylated proteins were revealed with the Transcend translation detection system (Promega) using peroxidase-coupled Streptavidin (1:5000) and a chemiluminescent substrate.

Microinjections

Xenopus laevis embryos were staged according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1967) and injected according to standard procedures (Sive et al., 2000). Capped mRNAs were synthesized with Message Machine Kit (Ambion). mRNAs and MOs were injected into single blastomeres at the two- to eight-cell stage that give rise to the dorsal ectoderm using the following amounts: *Dlx3*, 50-100 pg; *Dlx5*, 50-100 pg; *GATA2*, 50-250 pg; *GATA3*, 250 pg; *EnR-Dlx3hd*, 50 pg; *GATA2znf-EnR*, 50-100 pg; *FGF8*, 250 pg; Dlx3 MO, 20 ng; GATA2 MO, 4-10 ng; control MO, 20 ng. For rescue experiments, Dlx3 MO (20 ng) was co-injected with *Dlx5* (50 pg), while GATA2 MO (10 ng) was co-injected with *GATA3* (100-125 pg). Co-injection of *myc-GFP* (125-250 pg) or *lacZ* (250 pg) identified the injected side.

Pharmacological treatments

Embryos were unilaterally injected with *Dlx3* or *GATA2* mRNA and, after removal of vitelline membranes, were incubated in various signaling agonists or antagonists from stage 12.5 for 2.5 hours at room temperature in the dark. The following concentrations were used: dorsomorphin (Calbiochem), 100 μM (diluted from 1:100 stock in DMSO); SU5402 (Calbiochem), 60 μM (diluted from 1:1000 stock in DMSO); azakenpaullone (Sigma), 1 μM (diluted from 1:100 stock in DMSO). Control embryos were incubated in 1:100 DMSO.

Tissue grafts

Grafts were taken from pigmented donors or donors labeled by injecting *myc-GFP* (125 pg) into each blastomere at the four-cell stage and transplanted into albino or uninjected hosts, respectively, as previously described (Ahrens and Schlosser, 2005). For grafts of stage 13 neural plate into stage 13 belly ectoderm, the central anterior neural plate (see Ahrens and Schlosser, 2005) was placed into ventral ectoderm adjacent to the remnant of the blastocoel (Fig. 1). Marker expression was then analyzed at stages 18-26. Animal caps (stages 9-10), or prospective belly or neural plate ectoderm from various stage donors (stages 11-13) was grafted into the prospective neural crest and placode-forming region of the lateral neural plate border (NB) of stage 12-13 hosts (Fig. 2A). Prospective belly ectoderm was taken from the ventralmost region of the embryo overlying the residual blastocoel, whereas neural ectoderm was taken from the central anterior neural plate (see Ahrens and Schlosser, 2005). The expression of *Sox3*, *FoxD3* and *Six1* was then analyzed at stages 18-26. Only grafts that covered part of the neural crest (for *FoxD3*, *Sox3*) or panplacodal regions (for *Six1*) were analyzed.

In situ hybridization and immunohistochemistry

Whole-mount in situ hybridization was performed as previously described (Schlosser and Ahrens, 2004) using digoxigenin-labeled antisense probes for *Sox3* (Penzel et al., 1997), *Sox2* (de Robertis et al., 1997), *Sox11* (from T. Grammer and R. Harland, UCB, Berkeley, CA, USA), *Zic1* (Kuo et al., 1998), *Pax3* (Bang et al., 1997), *Msx1* (Su et al., 1991), *FoxD3* (Sasai et al., 2001), *Snail2* (Mayor et al., 1995), *Sox9* (Spokony et al., 2002), *Pax6* (Hollemann et al., 1998), *Pax2* (Heller and Brändli, 1997), *Pax8* (Heller and Brändli, 1999), *Eya1* (David et al., 2001), *Six1* (Pandur and Moody, 2000), *Foxi1* (Pohl et al., 2002), *Dlx3* (Luo et al., 2001b), *Dlx5* (Luo et al., 2001b), *GATA2* (Walmsley et al., 1994), *Keratin* (Jonas et al., 1989), *N-Tubulin* (Oschwald et al., 1991). Vibratome sections (30 μm) were prepared after whole mount in situ hybridization (Schlosser and Ahrens, 2004). myc-GFP and Sox3 were revealed immunohistochemically in wholemounts or sections using anti-c-myc (9E10, Developmental Studies Hybridoma Bank; 1:300) and anti-Sox3 (Zhang et al., 2004) (1:1000) primary antibodies and anti-mouse-Alexa488 or anti-rabbit-Alexa594 conjugated secondary antibodies (Invitrogen; 1:500 each), respectively, as previously described (Schlosser and Ahrens, 2004; Ahrens and Schlosser, 2005). Nonspecific binding of secondary antibodies was not observed when primary antibodies were omitted in control reactions.

RESULTS

Induction of panplacodal and neural crest markers at neural plate boundaries

We have previously reported that anterior neural plates from stage 13 donors maintain *Sox3* expression when grafted into belly ectoderm of same stage hosts (see also Fig. 1A), indicating that they are neurally committed and that they induce panplacodal markers *Six1* (see also Fig. 1F) and *Eya1* exclusively in non-neural host ectoderm but not in the neural graft (Ahrens and Schlosser, 2005). We now performed additional grafts of stage 13 neural plates into belly ectoderm to analyze induction of other neural plate border markers. Grafted neural plates typically invaginate to form miniature neural tubes and neural crest-like migratory cells (Fig. 1A-F).

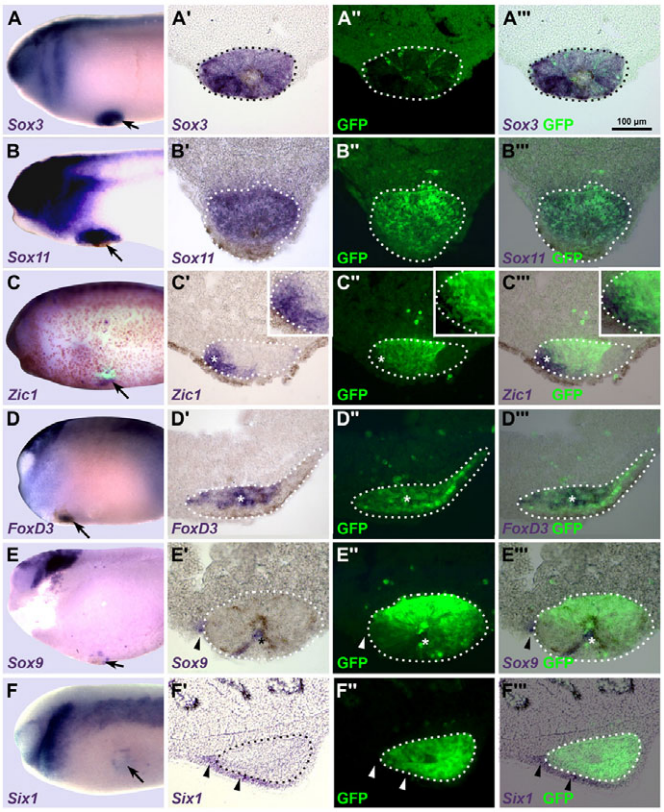

Fig. 1. Induction of ectodermal markers at neural plate boundaries. Ectodermal marker expression in tailbud stage embryos (albino) that had received neural plate grafts (pigmented and/or mycGFP-labeled) into belly ectoderm at stage 13. (**A-F‴**) Grafts (arrows, outlined) are shown in overviews (A-F) and transverse sections in brightfield (A′-F′), green fluorescent channel (A″-F″), and overlay (A‴-F‴). *Sox3* (A-A‴), *Sox11* (B-B‴), *Zic1* (C-C‴; inset shows higher magnification of expression in graft) and *FoxD3* (D-D‴) are maintained or induced (asterisks) in graft. *Six1* (F-F‴) is induced in host ectoderm (arrowheads), whereas *Sox9* (E-E‴) is induced in the graft (asterisk) and sometimes in host ectoderm (arrowhead).

Grafted neural plates maintain expression of neural (*Sox11*, 3/3) or anterior neural markers (*Pax6*, 8/8; *Pax2*, 6/6) (Fig. 1B, supplementary material Fig. S1A,C), whereas none of these are induced in the adjacent non-neural host ectoderm. Neural plate border genes such as *Zic1* (10/10) and *Pax3* (8/8) are expressed in grafted central neural plates (Fig. 1C, supplementary material Fig. S1B) but never in non-neural host ectoderm, suggesting that they can be induced in neural, but not in non-neural ectoderm. However, *Zic1* expression in the neural plate extends relatively far medially. Therefore, some of our grafts may have included *Zic1*-expressing parts of the neural plate so that *Zic1* was maintained rather than induced in these grafts. Similarly, the neural crest specifier genes *FoxD3*, *Snail2* and *Sox9* are induced in grafted neural plates (*FoxD3*, 10/12; *Snail2*, 3/21; *Sox9*, 7/11), whereas *FoxD3* or *Snail2* induction in non-neural host ectoderm was never observed (Fig. 1D,E). *Sox9*, which is expressed in the otic placode in addition to neural crest, was additionally induced in non-neural ectoderm in one case (1/11; Fig. 1E). However, other markers with placodal expression domains were not induced in non-neural ectoderm [*Sox3*, 0/9 as reported by Ahrens and Schlosser (Ahrens and Schlosser, 2005); *Sox11*, 0/3; *Pax6*, 0/8; *Pax3*, 0/8; *Pax2*, 0/6; *Pax8*, 0/5; supplementary material Fig. S1A-C)].

Our findings show that neural plate border specifiers, neural crest specifiers and some panplacodal markers can be induced at ectopic neural plate boundaries. However, neural crest and panplacodal markers are induced at opposite sides of the boundary, suggesting that the competence for expressing neural border/crest and panplacodal markers is confined to neural and non-neural ectoderm, respectively.

Differential distribution of competence for panplacodal and neural crest induction in non-neural and neural ectoderm

We next tested for differences in competence more directly by grafting neural or non-neural ectoderm into the lateral neural plate border (NB), i.e. the proper inducing environment for neural crest and pre-placodal ectoderm. We analyzed whether neural crest specifiers (*FoxD3*), neural markers (*Sox3*) or panplacodal markers (*Six1*) can be induced in belly ectoderm or central anterior neural plate ectoderm when grafted from stage 13 donor embryos into the NBs of stage 12-13 hosts (Fig. 2A).

We first established at which stage NBs provide an optimal signaling environment for panplacodal and neural crest induction. Previous experiments had shown that *Six1* can be reliably induced, when competent ectoderm (e.g. stage 13 belly ectoderm) is grafted into stage 13 NB (Ahrens and Schlosser, 2005). We confirm this here and show that stage 12 NB is less conducive for inducing *Six1* (Fig. 2L). By contrast, we find that *FoxD3* is more frequently induced, when competent ectoderm (e.g. stage 12 neural plate or belly ectoderm) was grafted into stage 12 rather than stage 13 NB (Fig. 2L,M). *Sox3* can be induced in ectoderm grafted into stage 12 or stage 13 NB (although more frequently in the former; Fig. 2L). However, *Sox3* is induced only in subregions of grafts (adjacent to retina and neural tube) when placed into 12 NB but throughout the grafts when placed into 13 NB (Fig. 2K), suggesting that inducing signals in 12 NB are more locally confined. To test for ectodermal competence in optimally inducing environments, we surveyed for *FoxD3* and *Sox3* induction in grafts that had been placed into stage 12 NB, and for *Six1* induction in grafts that had been placed into stage 13 NB.

When stage 13 neural plates are grafted into the NB, neither *Six1* (Fig. 2B,O) nor *Eya1* (0/11, not shown) are ever induced, whereas *FoxD3* is frequently induced (Fig. 2C,O) and *Sox3* maintained in at least part of the graft (Fig. 2D,O). By contrast, when stage 13 belly ectoderm is grafted into the NB, *Six1* is induced in all grafts (Ahrens and Schlosser, 2005) (see Fig. 2E,N), whereas neither *FoxD3* (except for 1/10; Fig. 2F,N) nor *Sox3* (Fig. 2G,N) can be induced in the graft. Taken together, this shows that at the end of gastrulation (stage 13), neural ectoderm is competent to express neural crest but not panplacodal markers, whereas non-neural ectoderm is competent to express panplacodal but not neural crest markers. However, dedicated placodal markers *Pax6* (0/11), *Pax3* (1/5), *Pax8* (1/7) and *Pax2* (0/9) were typically not induced in stage 13 belly ectoderm grafted into stage 13 NB (supplementary material Fig. S1D-H).

Establishment of differential competence between non-neural and neural ectoderm during gastrulation

To analyze when differences in ectodermal competence are established, we next grafted ectoderm from different stage donors into the NBs of stage 12 (*FoxD3* and *Sox3*) or stage 13 (*Six1*) hosts.

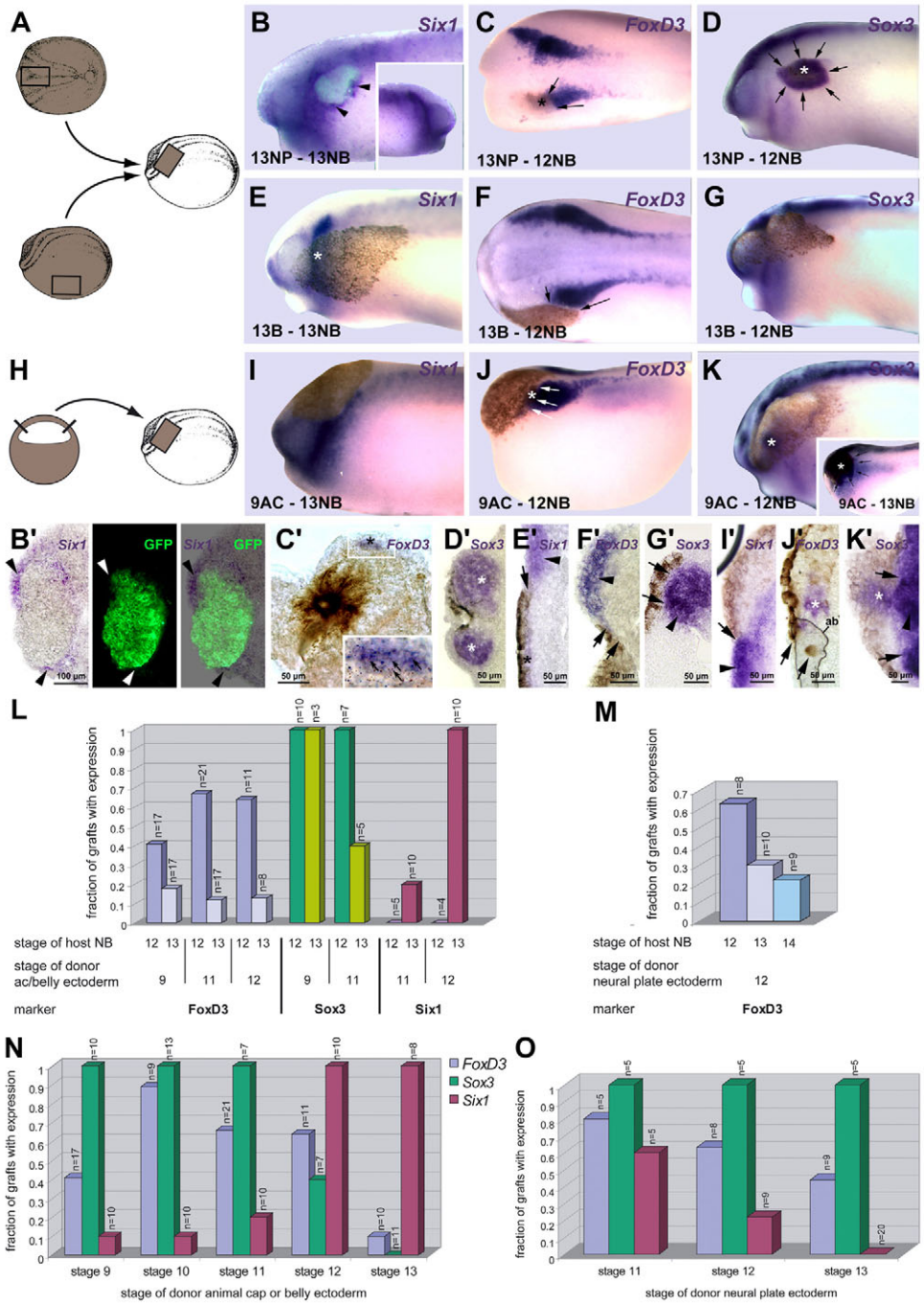

Fig. 2. Distribution of competence for neural crest and panplacodal induction. (**A-K′**) Experimental overview. After grafting mycGFP-labeled or pigmented ectoderm from various locations and stages into the lateral neural plate border (NB) of stage 12-13 hosts (A,H), expression of panplacodal (*Six1*), neural crest (*FoxD3*) or neural (*Sox3*) markers was analyzed in tailbud stage embryos (B-K). (B′-K′) Sections through grafts. Arrows highlight graft borders. Asterisks indicate expression in graft, whereas arrowheads indicate expression adjacent to graft. (B-D′) After grafting stage 13 neural plate (NP) into the NB, *FoxD3* but not *Six1* is induced and *Sox3* is maintained in the graft (inset in B, control side; inset in C′, magnified view of boxed area, arrows indicate pigment granules). (E-G′) After grafting stage 13 belly ectoderm (B) into the NB, *Six1* but neither *FoxD3* nor *Sox3* is induced in the graft. (I-K′) After grafting stage 9 animal caps (AC) into the NB, *FoxD3* and *Sox3* but not *Six1* are induced in the graft (inset in K, *Sox3* is more widely induced in grafts placed into stage 13 NB; ab in J′, air bubble). (**L**,**M**) Comparison of *FoxD3*, *Sox3* and *Six1* induction after grafting belly (L) or neural plate ectoderm (M) into NBs of different stage hosts. Stage 12 and stage 13 NB are more conducive to *FoxD3* and *Six1* induction, respectively. (**N**) Decline of *FoxD3* and *Sox3* induction, and increase in *Six1* induction with increasing age of belly ectoderm grafted into the NB. Data for *Six1* at stage 13 taken from Ahrens and Schlosser (Ahrens and Schlosser, 2005). (**O**) Decline of *Six1* induction with increasing age of neural plate ectoderm grafted into the NB.

We first grafted prospective belly ectoderm from different stage donors into the NB. At stages 9 and 10, prior to the onset of gastrulation, the grafts (animal cap ectoderm) were induced to express *FoxD3* (Fig. 2J,N) and *Sox3* (Fig. 2K,N), but almost never *Six1* in the NB (Fig. 2I,N). *FoxD3* was induced more rarely in stage 9 than in stage 10 animal caps. The fraction of grafts in which *FoxD3* could be induced then declines throughout gastrulation (stages 10-13; Fig. 2N). The fraction of grafts expressing *Sox3* similarly declines, whereas the fraction of grafts expressing *Six1* increases during these stages (Fig. 2N).

This suggests: (1) that prior to gastrulation ventral ectoderm, which is fated to become epidermis, is competent to give rise to other non-neural (panplacodal) as well as to neural (neural plate and neural crest) fates; (2) that in an environment where inducing signals for both neural (*Sox3*, *FoxD3*) and non-neural (*Six1*) fates are present, such as the NB, this ectoderm more readily adopts neural fates; and (3) that at the end of gastrulation ventral ectoderm loses the competence to adopt neural but remains competent to adopt non-neural fates.

Conversely, to test when dorsal ectoderm loses the competence to adopt non-neural fates, we next grafted prospective neural plate from stage 11 and 12 donors into the NB. We find that from stage 11 onwards, the prospective neural plate maintains *Sox3* expression in at least part of the graft. At stage 11, most prospective neural plates can also be induced to express both *FoxD3* and *Six1* after grafting to the NB. However, only the competence to express *FoxD3* persists until the end of gastrulation, whereas the competence to upregulate *Six1* disappears (Fig. 2O). This indicates

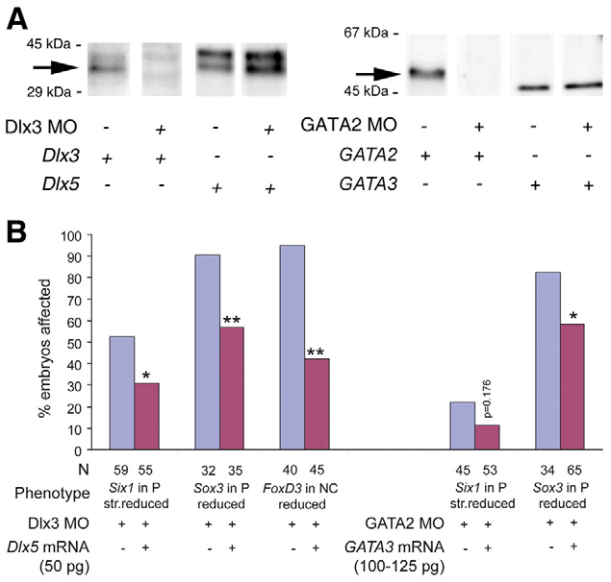

Fig. 3. Verification of efficacy and specificity of Dlx3 and GATA2 morpholinos. (**A**) Western blots after in vitro transcription and translation of Dlx and GATA proteins in presence or absence of Dlx3 MO or GATA2 MO. Dlx3 MO specifically reduces Dlx3 but not Dlx5 protein levels and GATA2 MO specifically reduces GATA2 but not GATA3 protein levels. (**B**) Co-injection of *Dlx5* mRNA with Dlx3 MO and of *GATA3* mRNA with GATA2 MO partially rescues effects of the MOs on placodes (P) and neural crest (NC) (Fisher's exact test; $*P \leq 0.05$, $**P \leq 0.01$). Because mild effects on placodal *Six1* expression were difficult to evaluate, we scored only embryos with strong defects. Probably owing to the low frequency of the latter after GATA2 MO injection, the rescue of placodal *Six1* expression was not statistically significant.

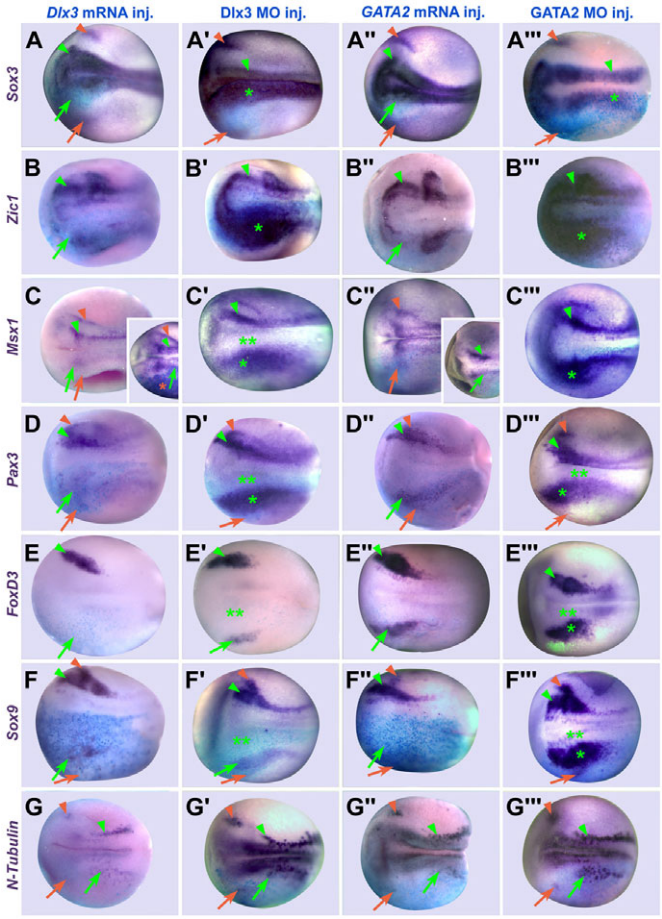

Fig. 4. Effects of Dlx3 or GATA2 gain or loss of function on neural ectodermal markers. (**A-G‴**) Neural plate stage embryos after unilateral injection (lower half; marked by light blue β-galactosidase or green mycGFP staining) of various constructs as indicated. Reductions (arrows), and broadening or ectopic expression domains (asterisks) in the neural (green) and non-neural ectoderm (orange) compared with the control side (arrowheads) are indicated. Double asterisks indicate lateral displacement owing to widening of the neural plate. For *Msx1*, additional examples of embryos are shown in the insets in C and C″.

that the loss of competence to acquire non-neural (e.g. placodal) fates in dorsal ectoderm coincides with the completion of neural induction at the end of gastrulation.

Role of Dlx3 and GATA2 transcription factors in panplacodal and neural crest induction

The expression patterns of *Dlx3* and *GATA2* suggest that they may be involved in promoting non-neural and inhibiting neural competence (supplementary material Fig. S2). Prior to gastrulation, *Dlx3* is co-expressed with *Sox3* throughout the ectoderm. During gastrulation, *Dlx3* becomes gradually restricted to prospective non-neural ectoderm in a complementary pattern to *Sox3*, which becomes neurally restricted. At neural plate stages, expression of *Dlx3* is then confined to non-neural ectoderm, excluding neural plate and neural crest. *GATA2* is expressed from stage 10 in a pattern that mirrors *Dlx3* expression.

These spatiotemporal changes of *Dlx3*/*GATA2* and *Sox3* expression correspond closely to changes in non-neural and neural competence, respectively. Indeed, *Dlx3* and *GATA2* are known to be required for adoption of non-neural ectodermal fates (Woda et al., 2003; Kaji and Artinger, 2004; Esterberg and Fritz, 2009; Kwon et al., 2010). However, both genes are also required for neural crest induction, raising questions about their precise roles at the neural plate border. To address this, we microinjected *Dlx3* and *GATA2* mRNAs or morpholinos (MOs) into one blastomere of four- to eight-cell stage embryos and subsequently studied the effect of their gain or loss of function on expression of neural plate markers (*Sox3*), neural plate border specifiers (*Zic1*, *Msx1*, *Pax3*), neural crest specifiers (*FoxD3*, *Sox9*), non-neural markers (*Dlx3*, *Dlx5*, *GATA2*), panplacodal genes (*Six1*, *Eya1*, *Foxi1*), dedicated placodal markers (Pax genes), epidermal markers (*Keratin*) and neuronal differentiation markers (*N-Tubulin*) in neural plate stage embryos.

The specificity and efficacy of MOs was verified by several approaches. First, we confirmed that Dlx3 MO blocks Dlx3 but not Dlx5 protein synthesis, whereas GATA2 MO blocks GATA2 but not GATA3 protein synthesis in vitro (Fig. 3A). Second, to confirm the MO data and to block additional and potentially functionally redundant Dlx and GATA family members, we injected *EnR-Dlx3hd* or *GATA2znf-EnR*, respectively. Both Dlx3 and GATA2 act as transcriptional activators, and these repressor constructs block transcription of target genes of Dlx3 and related Dlx proteins or GATA2 and related GATA proteins, respectively (Woda et al., 2005; Sykes et al., 1998). The phenotypes obtained (supplementary material Fig. S3) were similar (although sometimes more severe) to those observed after injection of Dlx3 MO and GATA2 MO

Table 1. Changes in marker gene expression in the neural ectoderm after injection of various constructs

	Dlx3 mRNA % (*n*)	*EnR-Dlx3hd* mRNA % (*n*)	Dlx3 MO¶ % (*n*)	*GATA2* mRNA % (*n*)	*GATA2znf-EnR* mRNA % (*n*)	GATA2 MO¶ % (*n*)	Control MO % (*n*)
***Six1*‡**							
Increased/ectopic	39 (72)	0 (50)	0 (124)	11 (71)	0 (40)	0 (79)	0 (38)
***Eya1*‡**							
Increased/ectopic	14 (79)	0 (42)	0 (65)	7 (28)	0 (12)	0 (47)	0 (45)
***FoxI1*‡**							
Increased/ectopic	12 (69)	0 (21)	0 (67)	0 (69)	0 (14)	0 (43)	0 (20)
***Dlx5*‡**							
Increased/ectopic	24 (45)	0 (32)	0 (44)	0 (26)	0 (29)	0 (48)	0 (24)
***Dlx3*‡**							
Increased/ectopic	nd	nd	nd	0 (49)	0 (26)	0 (51)	0 (32)
***GATA2*‡**							
Increased/ectopic	4 (79)	0 (39)	0 (106)	nd	nd	nd	0 (25)
***Keratin*‡**							
Increased/ectopic	3 (95)	0 (89)	0 (36)	0 (92)	16 (31)	0 (35)	0 (25)
***Sox3*§**							
Reduced	71 (49)	18 (61)	0 (64)	31 (88)	19 (47)	0 (34)	0 (60)
Increased/ectopic	0 (49)	70# (61)	94**# (64)	0 (88)	70# (47)	53**# (34)	0 (60)
Zic1							
Reduced	65 (68)	33 (27)	4 (50)	27 (33)	6 (34)	6 (32)	0 (20)
Increased/ectopic	1 (68)	33 (27)	80 (50)	21 (33)	44 (34)	91** (32)	0 (20)
***Msx1*§**							
Reduced	61 (62)	80 (20)	48** (56)	35 (31)	18 (50)	0 (27)	0 (25)
Increased/ectopic	31 (62)	0 (20)	36** (56)	0 (31)	26 (50)	78** (27)	0 (25)
***Pax3*§**							
Reduced	59 (73)	57 (14)	49** (57)	29 (42)	35 (43)	50** (46)	18 (28)
Increased/ectopic	0 (73)	14 (14)	26** (57)	0 (42)	40 (43)	45** (22)	0 (28)
FoxD3							
Reduced	53 (59)	93 (27)	78** (69)	45 (88)	21 (57)	62** (42)	5 (21)
Increased/ectopic	28 (39)	0 (27)	0 (69)	18 (88)	39 (57)	29** (42)	0 (21)
***Sox9*§**							
Reduced	74 (23)	74 (23)	57** (47)	79 (29)	13 (24)	19 (27)	4 (25)
Increased/ectopic	0 (23)	0 (23)	23** (47)	0 (29)	54 (24)	59** (27)	0 (25)
***N-Tubulin*§**							
Reduced	75 (36)	85 (20)	72** (74)	74 (47)	43 (40)	84** (83)	0 (32)
Increased/ectopic	0 (36)	0 (20)	0 (74)	0 (47)	0 (40)	0 (83)	0 (32)
***Pax6*§**							
Reduced	42 (38)	71 (21)	28** (18)	9 (22)	0 (28)	0 (18)	0 (24)
Increased/ectopic	0 (38)	0 (21)	39** (18)	0 (22)	0 (28)	61** (18)	0 (24)
***Pax8*‡**							
Increased/ectopic	0 (15)	0 (26)	nd	nd	nd	0 (21)	0 (48)

%, percent of embryos displaying phenotype; *n*, number of embryos analyzed; nd, not determined.
‡Gene not (or only weakly) expressed in neural ectoderm but was scored for increased/ectopic expression.
§Only expression in the neural ectoderm is considered here; for non-neural expression domains (epidermis or placodes), see Table 2.
¶Significant differences (Fisher's exact test; *P<0.05, **P<0.01) compared with control MO injections are indicated; in addition to the effects noted, there was lateral displacement of all markers.
#Domain of expression broader but no ectopic expression.

(Tables 1, 2; Figs 4, 5). Third, we demonstrated that non-specific control MOs had no effects (Tables 1, 2). Fourth, the effects of Dlx3 MO and GATA2 MO could be partially rescued by co-injection of *Dlx5* or *GATA3* mRNA, respectively (Fig. 3B). *Dlx5* and *GATA3* are expressed similar to *Dlx3* and *GATA2*, respectively, and have similar overexpression phenotypes (data not shown), suggesting that they can at least partially functionally compensate for each other.

DEVELOPMENT

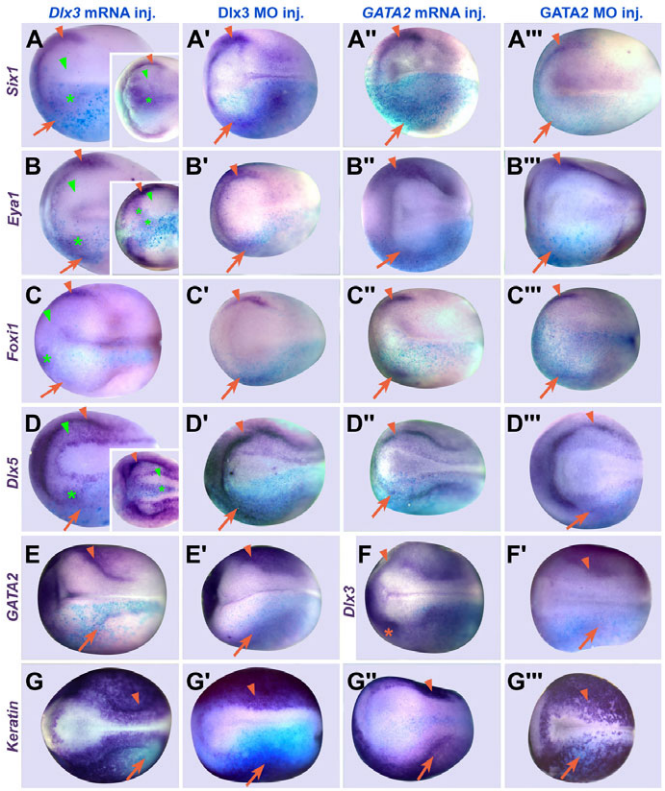

Fig. 5. Effects of Dlx3 or GATA2 gain or loss of function on non-neural ectodermal markers. (**A-G‴**) Neural plate stage embryos after unilateral injection (lower half; marked by light blue β-galactosidase or green mycGFP staining) of various constructs as indicated. Reductions (arrows) and broadening or ectopic expression domains (asterisks) in the neural (green) and non-neural (orange) ectoderm compared with the control side (arrowheads) are indicated. Insets depict additional embryos with ectopic expression of *Six1* (A), *Eya1* (B) and *Dlx5* (D) in central neural plate.

Our findings are summarized in Tables 1 and 2, which compile effects on marker gene expression in neural and non-neural ectoderm, respectively, and in Figs 4 and 5 and supplemental material Fig. 3. Gain of function of Dlx3 or GATA2 represses *Sox3* in the neural plate, as well as all neural plate border genes (*Zic1*, *Pax3*, *Msx1*). The boundary of the neural plate is typically not shifted in gain-of-function experiments, except for occasional minor medial or lateral displacements (Fig. 4, Table 1). By contrast, loss of function of Dlx3 and GATA2 resulted in broadening of neural plate (*Sox3*) and neural plate border markers (*Zic1*, *Pax3* and *Msx1*) and lateral displacement of all other markers (Fig. 4, Table 1). Levels of neural plate border genes within this broadened neural plate border region are increased in some embryos but reduced in others. This suggests that Dlx3 and GATA2 repress neural plate and neural plate border markers, precluding their expression in non-neural ectoderm. However, as relief of this repression results only in a lateral shift of the neural plate border rather than in ectopic expression of neural markers even after co-injection of Dlx3 MO/GATA2 MO (11/26 with lateral shift of *Sox3*) or *EnR-Dlx3hd*/*GATA2-znf-EnR* (17/23 with lateral shift of *Sox3*), additional factors in the ventral ectoderm probably functionally compensate for loss of Dlx or GATA.

The neural crest specifiers *FoxD3* and *Sox9*, as well as neuronal differentiation genes (*N-Tubulin*) are similarly reduced after overexpression of Dlx3 or GATA2 (with *FoxD3* being only mildly reduced after GATA2 overexpression) (Fig. 4, Table 1). After Dlx3 or GATA2 loss of function, the levels of these neural crest specifier and neuronal differentiation genes are also reduced while being expressed in a broadened expression domain with more pronounced reductions after Dlx3 loss of function (Fig. 4, Table 1). This suggests that Dlx3 and GATA2 are capable of repressing neural crest formation and neuronal differentiation in neural ectoderm, but that they (and in particular Dlx3) are also required in the non-neural ectoderm for proper neural crest formation and neuronal differentiation in the adjacent neural plate border region, possibly by regulating the production of inducing signals.

All markers of the non-neural ectoderm, including general non-neural (*Dlx3*, *Dlx5*, *GATA2*), panplacodal (*Six1*, *Eya1*, *FoxI1*), dedicated placodal (*Pax6*, *Pax3*, *Pax8*, *Sox9*), epidermal (*Keratin*) and neuronal markers (*N-Tubulin*) as well as *Sox3* expression in the pre-placodal ectoderm are reduced after Dlx3 or GATA2 loss of function (Figs 4, 5, Table 2). This suggests that Dlx3 and GATA2 are each required for normal development of non-neural ectoderm. Conversely, after Dlx3 or GATA2 overexpression, expression of *Msx1* and *Dlx3*, respectively, is often increased in the non-neural ectoderm, and panplacodal markers and *Keratin* are occasionally expressed more broadly. However, overexpression of Dlx3 or GATA2 mostly represses non-neural markers in the non-neural ectoderm (Fig. 5, Table 2). By contrast, overexpression of Dlx3 leads to upregulation of panplacodal (*Six1*, *Eya1*, *FoxI1*), general non-neural (*Dlx5*) and rarely epidermal (*Keratin*) but not of dedicated placodal or neuronal markers in the neural plate (Fig. 5, Table 1). GATA2 overexpression only rarely upregulates *Six1* and *Eya1*, but none of the other markers in the neural plate (Fig. 5, Table 1). Ectopic expression of non-neural markers in the neural plate is often weak and confined to subregions of the Dlx3- or GATA2-overexpressing domain. Taken together, this suggests that Dlx3 and GATA2 are required for adoption of different non-neural fates and Dlx3 is able to promote non-neural fates ectopically in the neural plate. However, at abnormally high levels both proteins actually suppress non-neural marker expression. Whether this is due to dose-dependent effects, the promotion of several mutually repressing fates or other mechanisms still needs to be resolved.

Dlx3 and GATA2 as competence factors in the non-neural ectoderm

The weak and local upregulation of non-neural markers in the neural plate after Dlx3 and sometimes after GATA2 overexpression suggests that Dlx3 and GATA2 may act as competence factors, which promote non-neural gene expression cell-autonomously but only in conjunction with locally confined signals. To test this hypothesis, we first analyzed whether Dlx3 and GATA2 are required cell-autonomously for panplacodal induction in non-neural ectoderm. We grafted stage 13 neural plates from pigmented embryos into the belly ectoderm of embryos that had been unilaterally co-injected with *mycGFP* and either Dlx3 MO or GATA2 MO at the two- to eight-blastomere stage. Although *Six1* was induced by the graft in the surrounding epidermis in 5/7 and 4/7 cases, respectively, its expression was specifically suppressed or reduced in cells expressing high levels of Dlx3 MO or GATA2 MO (Fig. 6), indicating a cell-autonomous requirement for Dlx3 and GATA2 in non-neural ectoderm.

We next tested whether Dlx3 or GATA2 promote non-neural fates only in conjunction with specific signaling molecules. We reared embryos that had been injected with *Dlx3* or *GATA2* mRNA into one out of eight blastomeres to stage 12.5-13 and then incubated them in various signaling agonists or antagonists (Fig.

Table 2. Changes in marker gene expression in the non-neural ectoderm after injection of various constructs

	Dlx3 mRNA % (*n*)	*EnR-Dlx3hd* mRNA % (*n*)	Dlx3 MO¶ % (*n*)	*GATA2* mRNA % (*n*)	*GATA2znf-EnR* mRNA % (*n*)	GATA2 MO¶ % (*n*)	Control MO % (*n*)
Six1							
Reduced	57 (67)	64 (50)	83** (134)	49 (93)	50 (40)	80** (103)	8 (38)
Increased/ectopic	6 (31)	0 (50)	0 (124)	13 (93)	5 (40)	0 (79)	0 (38)
Eya1							
Reduced	44 (79)	62 (42)	72** (65)	39 (28)	67 (12)	74** (47)	4 (45)
Increased/ectopic	6 (79)	0 (42)	0 (65)	7 (28)	0 (12)	0 (47)	0 (45)
Foxl1							
Reduced	33 (69)	43 (21)	64** (67)	53 (88)	43 (14)	60** (43)	15 (20)
Increased/ectopic	0 (69)	0 (21)	0 (67)	6 (88)	7 (14)	0 (43)	0 (20)
Dlx5							
Reduced	58 (45)	66 (32)	55** (44)	43 (49)	45 (29)	52** (48)	13 (24)
Increased/ectopic	0 (45)	0 (32)	14 (44)	0 (49)	7 (29)	8 (48)	0 (24)
Dlx3							
Reduced	nd	nd	nd	10 (49)	77 (26)	73** (51)	0 (32)
Increased/ectopic	nd	nd	nd	39 (49)	0 (26)	0 (51)	0 (32)
GATA2							
Reduced	66 (79)	38 (39)	52** (106)	nd	nd	nd	0 (25)
Increased/ectopic	0 (79)	0 (39)	0 (106)	nd	nd	nd	0 (25)
Keratin							
Reduced	93 (95)	98 (89)	86** (36)	21 (92)	74 (31)	69** (35)	0 (25)
Increased/ectopic	0 (95)	0 (89)	0 (36)	26 (92)	0 (31)	0 (35)	0 (25)
Sox3§							
Reduced	45 (49)	?# (61)	69** (64)	33 (88)	13+?# (47)	85** (34)	12 (60)
Increased/ectopic	12 (49)	?# (61)	0 (64)	0 (88)	?# (47)	0 (34)	0 (60)
Zic1‡							
Increased/ectopic	0 (68)	0 (27)	0 (50)	0 (33)	0 (34)	0 (32)	0 (20)
Msx1§							
Reduced	52 (62)	?# (20)	?# (56)	58 (31)	?# (50)	?# (27)	8 (25)
Increased/ectopic	35 (62)	?# (20)	?# (56)	0 (31)	?# (50)	?# (27)	0 (25)
Pax3§							
Reduced	63 (73)	71 (14)	60** (57)	62 (39)	33 (24)	70** (46)	18 (28)
Increased/ectopic	0 (73)	0 (14)	0 (57)	0 (39)	0 (24)	0 (46)	0 (28)
FoxD3‡							
Increased/ectopic	0 (59)	0 (27)	0 (69)	0 (88)	0 (57)	0 (42)	0 (21)
Sox9§							
Reduced	65 (23)	70 (23)	70** (47)	85 (27)	38 (24)	92 (25)	13 (24)
Increased/ectopic	0 (23)	0 (23)	0 (47)	0 (27)	0 (24)	0 (25)	0 (24)
N-Tubulin§							
Reduced	94 (36)	80 (20)	92** (74)	81 (43)	71 (21)	81** (83)	13 (32)
Increased/ectopic	0 (36)	0 (20)	0 (74)	0 (43)	0 (21)	0 (83)	0 (32)
Pax6§							
Reduced	50 (38)	81 (21)	41* (17)	59 (22)	36 (28)	50** (18)	4 (24)
Increased/ectopic	0 (38)	0 (21)	0 (17)	0 (22)	0 (28)	0 (18)	0 (24)
Pax8							
Reduced	40 (15)	81 (26)	nd	nd	nd	81** (21)	15 (48)
Increased/ectopic	0 (15)	0 (26)	nd	nd	nd	0 (21)	0 (48)

%, percent of embryos displaying phenotype; *n*, number of embryos analyzed; nd, not determined.
‡Gene not expressed in non-neural ectoderm but was scored for increased/ectopic expression.
§Only expression in the non-neural ectoderm is considered here; for neural expression domains (neural crest or neural plate), see Table 1.
¶Significant differences (Fisher's exact test; *P<0.05, **P<0.01) to control MO injections are indicated; in addition to the effects noted there was lateral displacement of all markers.
#Changes in pre-placodal ectoderm were unanalyzable in most cases because widened neural expression extends as far lateral as to pre-placodal expression domains.

DEVELOPMENT

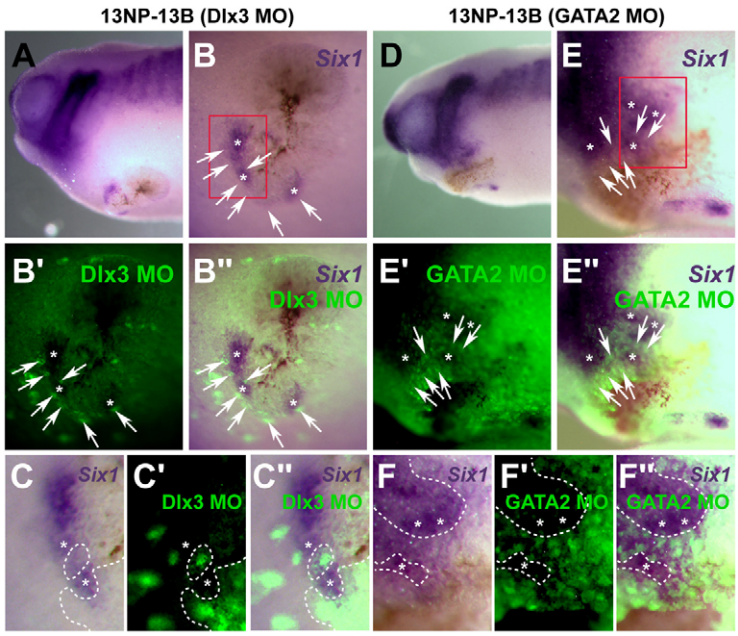

Fig. 6. Dlx3 and GATA2 are required cell-autonomously in the non-neural ectoderm for *Six1* induction. (**A-F″**) Host embryos were co-injected with *mycGFP* mRNA and either Dlx3 MO (A-C″) or GATA2 MO (D-F″) at the two- to eight-blastomere stage and received a neural plate (NP) graft (pigmented) into their belly ectoderm (B-B″) at stage 13. After host embryos reached tailbud stage, grafts are shown in overview (A,D) and at higher magnifications (B-C″,E-F″) in brightfield (B-F), green fluorescent channel (B′-F′) and an overlay (B″-F″). Boxed areas in B and E are shown in detail in C and F, respectively. *Six1* induction in non-neural host ectoderm around the graft (asterisks) is specifically suppressed in cells that received high levels of Dlx3 MO or GATA2 MO (arrows or outlined areas of green cells).

7). This allowed us to perturb signaling pathways selectively at neural plate stages, when panplacodal induction is known to commence (Ahrens and Schlosser, 2005).

Panplacodal induction has previously been shown to require inhibition of BMP and Wnt signaling, as well as FGF (Brugmann et al., 2004; Ahrens and Schlosser, 2005; Litsiou et al., 2005). We therefore tested first whether treatment of embryos with dorsomorphin, a selective inhibitor of BMP signaling (Yu et al., 2008), would expand *Six1* expression in *Dlx3*- or *GATA2*-injected embryos. Indeed, ectopic neural expression of *Six1* was drastically increased in frequency and intensity compared with DMSO-treated controls in *Dlx3* but not in *GATA2*-injected embryos and extended throughout the Dlx3-overexpressing region in the anterior neural plate (Fig. 7B,C). This upregulation could be blocked by azakenpaullone, an agonist of Wnt signaling (Kunick et al., 2004), or by SU5402, an antagonist of FGF signaling (Mohammadi et al., 1997), but was unaffected by co-injection of *FGF8* (Fig. 7C). This shows that FGF and Wnt inhibition are also required for panplacodal induction in *Dlx3*-expressing ectoderm and suggests that the signaling environment of the anterior neural plate, apart from providing insufficient levels of BMP antagonists, contains all other signals (FGF, Wnt antagonists) required for panplacodal induction.

We next tested whether *Dlx3*-expressing ectoderm adopts other non-neural fates in the absence of some of these inducers. Indeed, we observed strong ectopic epidermal *Keratin* expression in the *Dlx3*-overexpressing part of the neural plate in DMSO-treated controls, but never in dorsomorphin-treated embryos (Fig. 7A,C). Interestingly, ectopic *Keratin* expression was only rarely observed in *Dlx3*-injected embryos without DMSO treatment (Table 1), suggesting that DMSO itself modulates the signaling environment. Ectopic expression domains of non-neural markers (*Six1*, *Keratin*) in the neural plate were strictly confined to *Dlx3*-overexpressing regions, which were precisely complementary to residual Sox3 immunostaining (Fig. 7D,E), indicating that these markers can only be induced after conversion of prospective neural to non-neural ectoderm by Dlx3.

Our findings show that Dlx3 is cell-autonomously required for non-neural marker expression and is sufficient to repress neural fates and promote panplacodal or epidermal fates in the absence or presence of BMP, respectively, indicating that it confers non-neural competence on the ectoderm. GATA2, by contrast, is also cell-autonomously required for non-neural competence but is unable to promote it ectopically.

DISCUSSION

Mutually exclusive neural and non-neural competence territories are established during gastrulation

We show here that, prior to gastrulation, ectoderm is competent to generate all ectodermal fates, but during gastrulation the competence to form neural crest becomes restricted to dorsal ectoderm, whereas the competence to express the panplacodal marker *Six1* becomes ventrally restricted. These changes parallel the dorsal restriction of neural competence, suggesting that, during gastrulation, two mutually exclusive competence territories are established: a dorsal neural territory that has neural plate as a default state but can be induced to form neural crest; and a ventral non-neural territory that has epidermis as default state but can be induced to form pre-placodal ectoderm. Our data extend previous studies showing that ventral ectoderm loses competence to form neural plate or neural crest at the end of gastrulation, whereas the competence to form lens placodes increases (Servetnick and Grainger 1991; Kintner and Dodd, 1991; Kengaku and Okamoto, 1993; Mancilla and Mayor, 1996).

These findings confirm the predictions of the ‘binary competence model’ (Ahrens and Schlosser, 2005; Schlosser, 2006), but do not support models according to which a common neural plate border state is first established from which neural crest and placodes are subsequently induced (Streit and Stern, 1999; Baker and Bronner-Fraser, 2001; McLarren et al., 2003; Woda et al., 2003; Glavic et al., 2004; Meulemans and Bronner-Fraser, 2004; Brugmann et al., 2004; Litsiou et al., 2005; Patthey et al., 2008; Patthey et al., 2009).

Our experiments further show that although ectoderm from early gastrulae can form all ectodermal fates, it will preferentially differentiate into neural plate or neural crest rather than panplacodal fates if exposed to signals in the neural plate border region, indicating that neural competence overrules non-neural competence. This suggests, in line with the ‘neural default model’

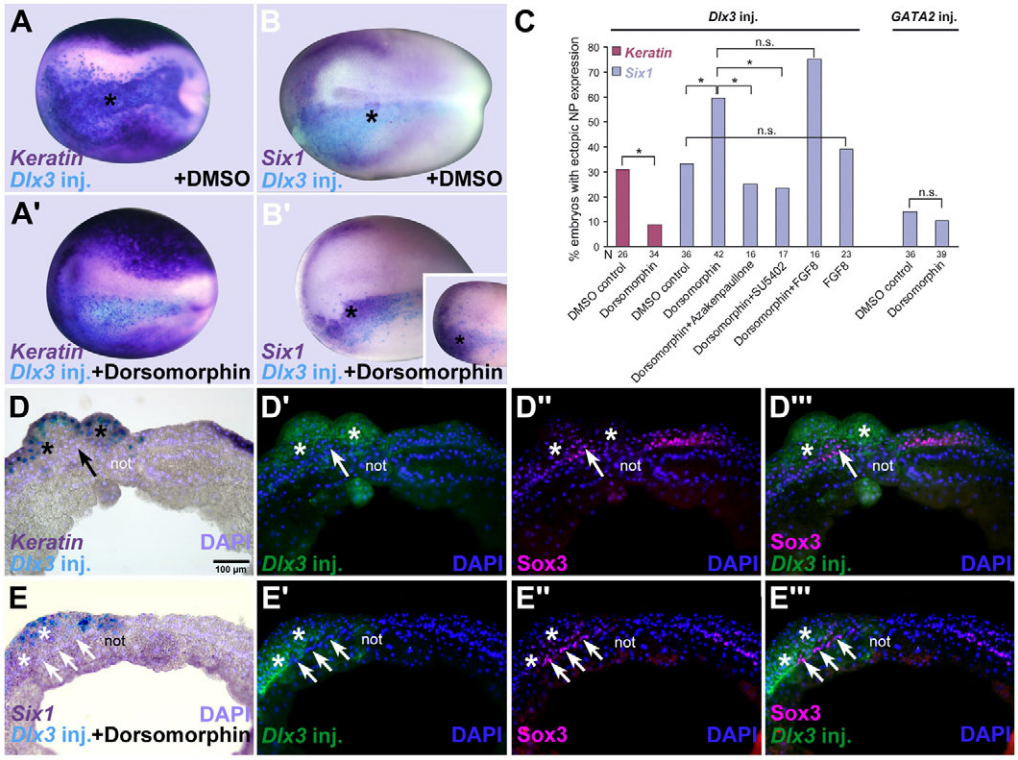

Fig. 7. Dlx3 promotes different non-neural fates depending on the signaling environment. (**A-B′**) *Keratin* (A,A′) and *Six1* (B,B′) expression in neural plate stage embryos after unilateral injection (lower half; marked by light-blue β-galactosidase staining) of *Dlx3* mRNA and treatment with DMSO (A,B) or the BMP signaling antagonist dorsomorphin (A′,B′). Ectopic *Keratin* expression in the neural plate is strongly reduced, while ectopic *Six1* expression is strongly enhanced by dorsomorphin treatment (inset in B′ shows another example). (**C**) Effects of signaling agonists or antagonists on ectopic expression of *Keratin* or *Six1* in the neural plate (NP). After *Dlx3* mRNA injection, the increase in ectopic *Six1* expression by dorsomorphin was blocked by the Wnt agonist azakenpaullone and the FGF antagonist SU5402, whereas co-injection of *FGF8* had no significant effect. After *GATA2* mRNA injection, dorsomorphin did not increase ectopic *Six1* expression, whereas *Keratin* (not shown) was never ectopically expressed (Fisher's exact test; *$P \leq 0.05$, n.s., not significant). (**D-E‴**) Transverse sections through neural plate of embryos shown in A (D-D‴) and the inset of B′ (E-E‴). Sections are shown in brightfield (D,E) in the green fluorescent channel, showing mycGFP-positive *Dlx3*-injected cells (D′,E′); in the red fluorescent channel, showing Sox3 immunostaining (D″,E″); and in overlay (D‴,E‴). DAPI-stained nuclei are shown for orientation in all panels. Ectopic *Keratin* and *Six1* expression is confined to *Dlx3*-injected cells (asterisks), whereas residual Sox3 staining on injected side is found only in cells that did not receive *Dlx3* (arrows). not, notochord.

(de Robertis and Kuroda, 2004; Vonica and Hemmati-Brivanlou, 2006), that neural competence is the default state. Taking into account the known role of BMPs in repressing neural fate and the establishment of a dorsoventral BMP gradient due to secretion of BMP inhibitors by dorsal midline tissues during gastrulation (reviewed by de Robertis and Kuroda, 2004; Stern, 2006; Vonica and Hemmati-Brivanlou, 2006), we therefore propose the following model (Fig. 8). In ventral ectoderm, BMP expression promotes the expression of non-neural competence genes (e.g. *Dlx3* and *GATA2*; see below), which repress neural competence genes. In dorsal ectoderm, BMP inhibition relieves this repression of neural competence genes. Transcriptional cross-repression between neural and non-neural competence factors ensures the formation of stable and mutually exclusive territories for neural and non-neural competence during gastrulation. At the beginning of gastrulation, the expression of neural and non-neural competence genes is still labile and BMP dependent, and the entire ectoderm, therefore, maintains neural as well as non-neural competence. Subsequently, however, their expression becomes stabilized and BMP independent, e.g. due to auto-activation. At neural plate stages, non-neural competence factors then promote transcription of epidermal or – in the presence of signals such as BMP inhibitors, FGFs and Wnt inhibitors – panplacodal genes, whereas neural competence factors promote transcription of neural plate genes or – in the presence of signals such as BMP, Wnt and FGF – neural crest genes.

In agreement with this model, it has recently been shown that induction of neural crest and neural plate requires low BMP levels during gastrulation (Steventon et al., 2009; Patthey et al., 2009), whereas induction of many ventral transcription factors, pre-placodal ectoderm and epidermis requires high BMP levels (Kwon et al., 2010). From neural plate stages onwards, the expression of many ventrally localized transcription factors becomes BMP independent (Kwon et al., 2010) and BMP requirements for neural crest and panplacodal induction change. BMP inhibition is now required for induction of pre-placodal ectoderm (Ahrens and Schlosser, 2005; Litsiou et al., 2005; Esterberg and Fritz, 2009; Kwon et al., 2010), whereas some BMP signaling is required for neural crest induction (Steventon et al., 2009; Patthey et al., 2008; Patthey et al., 2009). Because BMP signaling recedes from the neural plate border from stage 13 onwards (Schohl and Fagotto, 2002), these later phase BMP requirements also explain why stage 12 and stage 13 NBs provide better inducing environments for neural crest and panplacodal induction, respectively.

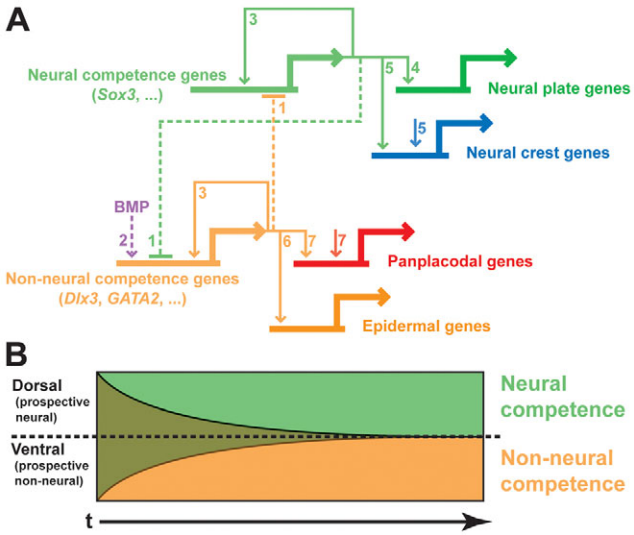

Fig. 8. Model for regulation of ectodermal competence. (**A**) Genes that promote non-neural competence, including *Dlx3* and *GATA2* (orange), and those that promote non-neural competence, probably including *Sox3* (green), cross-repress each others transcription (1, broken lines indicate indirect effects). Expression of non-neural competence genes is initially dependent on BMP signaling (2). In the presence of BMP, transcription of non-neural competence genes is therefore promoted over neural competence genes, whereas the reverse is true in the absence of BMP. However, persistent expression of these genes may lead to their autoactivation (3), thereby making their expression resilient to repression and BMP independent. Neural competence factors promote transcription of neural plate genes (4) or, in the presence of additional signals such as BMP, Wnt and FGF (5), neural crest genes. Non-neural competence factors promote transcription of epidermal genes (6) or, in the presence of additional signals such as BMP inhibitors, FGFs and Wnt inhibitors (7), panplacodal and placodal genes. (**B**) Owing to the dorsal secretion of BMP antagonists and crossrepressive interactions among competence genes, their initially overlapping expression domains will resolve into two distinct territories over time (t).

Role of Dlx3 and GATA2 as non-neural competence factors

Our loss-of-function experiments show that Dlx3 and GATA2 are required for the expression of epidermal, panplacodal and dedicated placodal markers, as well as for neural crest induction and the maintenance of neural plate border markers, confirming and significantly extending previous studies (McLarren et al., 2003; Woda et al., 2003; Kaji and Artinger, 2004; Esterberg and Fritz, 2009; Rossi et al., 2009; Kwon et al., 2010). However, neither Dlx3 nor GATA2 is expressed in the neural plate or neural crest territory, indicating that they promote neural crest or neural plate border development in a non-cell-autonomous manner (see also McLarren et al., 2003; Kaji and Artinger, 2004; Rossi et al., 2009), e.g. by promoting the production of inducing signals in non-neural ectoderm.

By contrast, the pattern of Dlx3 and GATA2 expression in the non-neural ectoderm matches closely the spatiotemporal distribution of non-neural competence, making them good candidates for the non-neural competence factors proposed in our model (Fig. 8). *Dlx3* and *GATA2* transcription is activated by BMP signaling, which accounts for the ventral restriction of these and other transcription factors (e.g. Vent2, FoxI1, AP2) during gastrulation (Read et al., 1998; Pera et al., 1999; Feledy et al., 1999; Luo et al., 2001a; Friedle and Knöchel, 2002). However, only GATA but not Dlx genes are direct BMP target genes (Feledy et al., 1999; Friedle and Knöchel, 2002). Moreover, although expression of Dlx and GATA genes initially requires BMP, they become BMP independent during gastrulation (Kwon et al., 2010), which could underlie the gradual stabilization of non-neural competence that our model predicts. Although it is currently not clear which transcription factors confer neural competence, the early onset of *Sox3* expression in the ectoderm, its gradual restriction to neural ectoderm in a complementary pattern to *Dlx3* and its ability to repress non-neural markers (including Dlx3 and GATA2) (Penzel et al., 1997; Rogers et al., 2008; Rogers et al., 2009) (G.S., unpublished) make it, at present, a most promising candidate.

Our findings also confirm previous reports that Dlx and GATA factors repress neural development (Xu et al., 1997; Shibata et al., 1998; Pera et al., 1999; Feledy et al., 1999; Woda et al., 2003; McLarren et al., 2003; Linker et al., 2009), supporting a role for Dlx3 and GATA2 in repression of neural competence. However, as Dlx and GATA factors are transcriptional activators, their repressive effects on neural development are probably indirect. Moreover, loss of function of Dlx3 and/or GATA2 only expands *Sox3* and other neural markers but does not lead to their ectopic expression in ventral non-neural ectoderm. This is also true after co-injection of repressor constructs, which broadly inhibit Dlx and GATA target genes, suggesting that additional and functionally partly redundant factors in the non-neural ectoderm contribute to repression of neural competence. One such ventrally restricted transcription factor is Vent2, which directly represses *Sox3* transcription, thereby restricting it to the neural plate (Rogers et al., 2008).

Although these findings suggest important roles for Dlx3 and GATA2 during development of non-neural ectoderm, three additional conditions have to be met to establish them as non-neural competence factors: (1) they should be cell-autonomously required in the non-neural ectoderm; (2) they should be sufficient to convert prospective neural ectoderm to non-neural ectoderm; and (3) they should promote different non-neural fates depending on the signaling pathways activated. We show here that both factors are cell-autonomously required for *Six1* induction in the non-neural ectoderm, as has been previously suggested for GATA3 in the zebrafish (Kwon et al., 2010), and, thus, meet condition one. We also find that Dlx3, but not GATA2, gain of function is sufficient to promote adoption of different non-neural fates in the prospective neural plate, depending on the signaling pathways co-activated. Induction of the panplacodal marker *Six1* is known to require FGF together with BMP and Wnt inhibition (Brugmann et al., 2004; Ahrens and Schlosser, 2005; Litsiou et al., 2005; Kwon et al., 2010). In agreement with this, Dlx3-overexpressing neural plates upregulated *Six1* when BMP signaling was blocked without concomitant inhibition of FGF or activation of Wnt signaling, whereas *Keratin* was upregulated in the presence of BMP. Thus, Dlx3, but not GATA2, also meets conditions two and three. The absence of strong upregulation of panplacodal or epidermal markers in the neural plate after overexpression of Dlx3 or Dlx5 in previous studies (McLarren et al., 2003; Woda et al., 2003) may therefore be due to the failure to co-activate the proper signaling pathways. Although GATA2 overexpression was not sufficient to promote non-neural fates in the *Xenopus* neural plate, GATA3 synergizes with FoxI1 and AP2 in conferring non-neural competence to the zebrafish neural plate (Kwon et al., 2010). It remains to be tested whether this also applies to *Xenopus*.

Our findings, therefore, strongly support a role for Dlx3 in the regulation of non-neural competence, while GATA2 may contribute to non-neural competence but is not sufficient to confer it ectopically. The molecular mechanisms by which Dlx and GATA factors promote non-neural competence remain to be elucidated. A recent study in zebrafish suggested that Dlx3 mediates competence by promoting expression of the BMP antagonist CV2, which makes ectoderm responsive to the panplacodal inducer FGF by relieving BMP-mediated repression of FGF receptors (Esterberg and Fritz, 2009). However, in zebrafish, CV2 expression is confined to the pre-placodal region itself, whereas non-neural competence is distributed throughout the entire ventral ectoderm (Kwon et al., 2010), suggesting that additional mechanisms must be involved.

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