

FGF/MAPK signaling is required in the gastrula epiblast for avian neural crest induction

Timothy J. Stuhlmiller and Martín I. García-Castro*

SUMMARY

Neural crest induction involves the combinatorial inputs of the FGF, BMP and Wnt signaling pathways. Recently, a two-step model has emerged where BMP attenuation and Wnt activation induces the neural crest during gastrulation, whereas activation of both pathways maintains the population during neurulation. FGF is proposed to act indirectly during the inductive phase by activating Wnt ligand expression in the mesoderm. Here, we use the chick model to investigate the role of FGF signaling in the amniote neural crest for the first time and uncover a novel requirement for FGF/MAPK signaling. Contrary to current models, we demonstrate that FGF is required within the prospective neural crest epiblast during gastrulation and is unlikely to operate through mesodermal tissues. Additionally, we show that FGF/MAPK activity in the prospective neural plate prevents the ectopic expression of lateral ectoderm markers, independently of its role in neural specification. We then investigate the temporal participation of BMP/Smad signaling and suggest a later involvement in neural plate border development, likely due to widespread FGF/MAPK activity in the gastrula epiblast. Our results identify an early requirement for FGF/MAPK signaling in amniote neural crest induction and suggest an intriguing role for FGF-mediated Smad inhibition in ectodermal development.

KEY WORDS: Neural crest induction, FGF, MAPK, Smad, Neural plate border, Gastrulation, Pax7, Chick

INTRODUCTION

The neural crest (NC) is a population of multipotent embryonic cells that migrates from the dorsal neural tube to give rise to a diverse array of derivatives, including melanocytes, sensory neurons of the peripheral nervous system, and most of the bone and cartilage of the face and skull. NC progenitors are first identifiable by the expression of several transcription factors immediately following gastrulation at the neural plate border (NPB), a collection of ectodermal cells flanked medially by the neural plate (NP) and laterally by the non-neural ectoderm (NNE), with a layer of mesoderm found underneath. The NC is thought to be formed through an inductive mechanism, whereby interactions between ectodermal tissues and the mesoderm bring about the formation of the NPB (Liem et al., 1995; Mancilla and Mayor, 1996; Moury and Jacobson, 1990; Raven and Kloos, 1945; Selleck and Bronner-Fraser, 1995). The precise participation of the different tissues, however, seems to be species specific. The mesoderm, for example, is crucial to *Xenopus* NC induction (Bonstein et al., 1998; Hong et al., 2008; Marchant et al., 1998; Monsoro-Burq et al., 2003; Steventon et al., 2009), yet it appears to be dispensable in zebrafish (Ragland and Raible, 2004).

Several extracellular signaling pathways have been implicated in NC induction, with most studies focusing on bone morphogenetic protein (BMP), fibroblast growth factor (FGF) and Wnt signaling (Chang and Hemmati-Brivanlou, 1998; García-Castro et al., 2002; LaBonne and Bronner-Fraser, 1998; Lewis et al., 2004; Liem et al., 1995; Mayor et al., 1997; Mayor et al., 1995; Nguyen et al., 1998; Saint-Jeannet et al., 1997; Selleck et al., 1998) [for further references, see Jones and Trainor (Jones and Trainor,

2005)]. Recent evidence from *Xenopus* and chick embryos supports a two-step model of NC induction, with an early phase requiring Wnt activation and BMP inhibition during gastrulation, followed by a later phase of both Wnt and BMP activation during neurulation (Patthey et al., 2009; Patthey et al., 2008; Steventon et al., 2009). Although *Xenopus* studies have identified the likely inductive molecules, their sources and the time at which they act, recent work in chick has only explored the timing. Furthermore, these experiments in chick used embryonic explants and lacked in vivo information on the presence or absence of Wnt and BMP activation throughout early development.

In contrast to BMP and Wnt signaling, the role of FGF signaling in NC induction has only been investigated in *Xenopus* (Hong et al., 2008; Hong and Saint-Jeannet, 2007; LaBonne and Bronner-Fraser, 1998; Mayor et al., 1997; Mayor et al., 1995; Monsoro-Burq et al., 2003; Monsoro-Burq et al., 2005; Villanueva et al., 2002). It is currently proposed that FGFs act on the paraxial mesoderm during gastrulation to bring about the expression of Wnt8, which then signals to the overlying ectoderm to induce the NC (Hong et al., 2008). Thus, FGF is thought to induce the NC indirectly through the mesoderm. Although the participation of FGF in NC induction has not been addressed in the chick, studies have identified an early requirement for FGF signaling in mesoderm induction (Bertocchini et al., 2004; Chuai et al., 2006; Storey et al., 1998), placode development (Adamska et al., 2001; Ladher et al., 2005; Litsiou et al., 2005), neural induction (Linker and Stern, 2004; Stavridis et al., 2007; Streit et al., 2000; Wilson et al., 2000; Wilson et al., 2001) and later development in the caudal neural plate/stem zone (Akai et al., 2005; Delfino-Machin et al., 2005; Diez del Corral et al., 2003; Olivera-Martinez and Storey, 2007). The induction of neural tissue, which occurs in close spatial and temporal proximity to the formation of the NPB, requires FGF/MAPK signaling up until the gastrula stage, both to antagonize BMP signaling and to act directly on the prospective neural ectoderm (Linker and Stern, 2004; Sheng et al., 2003; Stavridis et al., 2007; Streit et al., 2000; Wilson et al., 2000; Wilson et al., 2001).

Department of Molecular, Cellular and Developmental Biology, Yale University, New Haven, CT 06520-8103, USA.

* Author for correspondence (martin.garcia-castro@yale.edu)

Here, using the chick model, we provide the first in vivo evidence that FGF/MAPK signaling is required for NC induction in amniotes and provide a different perspective to indirect mesoderm-based induction. Using a truncated, dominant-negative form of Fgfr1, the general FGF receptor inhibitor SU5402 and a cell-autonomous MAPK inhibitor, we identify a novel requirement for FGF/MAPK signaling during gastrulation within the prospective NPB epiblast. In agreement, FGF receptors are expressed in the ectoderm but are not found in the mesoderm at the time of NC induction. We additionally find a different role for FGF/MAPK signaling in the prospective NP, where it prevents the ectopic expression of lateral ectoderm markers. Interestingly, this activity is independent of neural specification, suggesting that FGF/MAPK signaling has multiple separable roles in the development of ectodermal tissues. Given the established role of BMP signaling in NC induction, we analyze the temporal participation of Smad1/5/8 signaling, and provide in vivo evidence for its requirement after gastrulation. Last, we show that increased Smad1/5/8 signaling is likely to be responsible for the ectopic expression of lateral markers in NP tissues with attenuated FGF signaling. Our results identify a novel mechanism of FGF/MAPK action in NC induction, and suggest an important role for FGF/MAPK-mediated Smad inhibition in the early development of ectodermal tissues in amniotes.

MATERIALS AND METHODS

Expression constructs

The chick dominant-negative Fgfr1 sequence (a gift from S. Fraser, Caltech, Pasadena, CA, USA) was subcloned into pCIG after adding exogenous 5' *XhoI* and 3' *Clal* sites. Full-length chick Mkp3 (also known as Dusp6) (bp 147-1295, NM_204254) was cloned into pCIG after adding exogenous 5' *XhoI* and 3' *XmaI* sites. Both pCA β and cSmad6 pCA β were gifts from C. Stern (UCL, London, UK).

In situ probes were generated as follows: cFgfr mRNA sequences were cloned into pBluscript+ after addition of exogenous 5' *Acc65I* and 3' *SacI* sites. Sequences used: cFgfr1, bp 565-973 (NM_205510); cFgfr2, bp 606-975 (NM_205319); cFgfr3, bp 485-861 (NM_205509). *cBrachyury* bp 707-1139 (NM_204940) was cloned into pBluscript+ after addition of exogenous 5' *XhoI* and 3' *XbaI* sites.

Other probes were gifts: *cBmp4* (O. Pourquié, IGMBC, Strasbourg, France), *cGata2* (D. Engel, U-M, Ann Arbor, MI, USA), *cSox2* (A. Groves, BCM, Houston, TX, USA), *cWnt8c* (J. Dodd, Columbia University, NY, USA) and *cTbx6L* (S. Mackem, NCI, NIH, Frederick, MD, USA). *Fgfr4* was detected with a quail *FREK* probe (C. Marcelle, Monash University, Clayton, Victoria, Australia).

Embryos and electroporation

Fertile hen eggs were obtained from Hardy's Hatchery (Massachusetts, USA). Embryos were staged according to Hamburger and Hamilton (Hamburger and Hamilton, 1951), with embryos having morphology between stage 3+ and 4 given the stage designation '4-' (supplementary material Fig. S1).

DNA for electroporation was used at 1 mg/ml in 0.01% Fast Green in Milli-Q water. For focused electroporation, sucrose was added to a final concentration of 6% (w/v). Electroporation was carried out in Howard Ringers (HR) in chambers containing a platinum plate below the embryo while holding a platinum electrode above, with five pulses of 6V (50 ms on, 100 ms off). Embryos were cultured at 38°C for 16-20 hours in EC culture (Chapman et al., 2001).

Explant preparation and culture

The hypoblast of stage 3 embryos was mechanically removed using glass needles, and a horizontal strip of epiblast tissue was cut from the center of the embryo. This strip was trimmed to include only the area pellucida and dissected into 10 equivalent-sized squares (each approximately 100 μm^2) after discarding the primitive streak. These squares were cut in half diagonally and immobilized in separate collagen gels in four-well plates.

Collagen gels were prepared by combining 90 μl 3.68 mg/ml collagen (BD Biosciences), 10 μl of 10 \times DMEM (Gibco) and 3.7 μl of 7.5% sodium hydrogen carbonate. Collagen gels were immersed in DMEM/F12 containing N2 supplement (Gibco) and either SU5402 (5 or 10 μM) or DMSO for 16 hours at 37°C. Explants were fixed in 4% paraformaldehyde for 15 minutes before immunostaining.

Bead grafting experiments

Agarose beads were soaked in PBS+0.1%BSA containing SU5402 (Calbiochem) or an equivalent volume of DMSO for at least 1 hour at room temperature, then washed three times in PBS+0.1%BSA immediately before grafting. SU5402 concentrations of 100, 50 and 25 μM were used, and all yielded similar results, so the results were combined. Beads were grafted between the upper (epiblast or ectoderm) and lower (hypoblast or mesoderm) layers. Embryos were cultured for 16 hours when beads were grafted at gastrulation stages, and cultured for 2-7 hours if grafted at later stages. Pax7 mRNA and protein were assayed, and both gave similar results, so the data were combined.

Protein and mRNA detection

For immunofluorescence, embryos were fixed in freshly-thawed 4% paraformaldehyde (w/v) for 30-45 minutes at room temperature (except for phospho-specific antibody staining, see below), then rinsed in PBS+0.1% Tween (PT). For in situ hybridization, embryos were first imaged in HR using a SPOT SE camera and software using a Nikon SMZ 1500 microscope, and then fixed for 2 hours at room temperature or overnight at 4°C.

Whole-mount immunofluorescence and in situ hybridization were performed as previously described (Basch et al., 2006). Primary antibodies were diluted as follows: 1:50 Pax7 (mIgG1, Developmental Studies Hybridoma Bank), 1:1500 Snail2 (rbIgG, Cell Signaling #9585), 1:60 Sox2 (gtIgG, R&D Systems #AF2018) and 1:40 GFP (rbIgG, Millipore, #AB3080). Secondary antibodies (Alexa 488, 568, or 633, from Invitrogen) were used at 1:3000. Staining for dual-phosphorylated Erk1/2 [rbIgG Phospho-p44/42 MAPK (Thr202/Tyr204), Cell Signaling #9101] was carried out as follows: embryos were fixed in ice-cold paraformaldehyde at 4°C for 1.5 hours or overnight, rinsed in ice-cold PT, then dehydrated to methanol and placed at -20°C overnight. Embryos were rehydrated to PT and kept at 4°C for the remainder of the protocol. Embryos were blocked with PBTS (PBS+2% BSA+0.1% Tween+10% horse serum) overnight, incubated with dpERK antibody (1:50) in PBTS for 3-5 days, then washed with PT several times. Embryos were re-blocked for 30 minutes in PBTS, incubated with Biotinylated anti-rbIgG (1:200, Vector Labs #BA-1000) in PBTS overnight, then washed with PT several times. Embryos were re-blocked for 30 minutes in PBTS, incubated with Streptavidin-Alexa 568 conjugate (1:1500, Invitrogen #S-11226) in PBTS overnight, then washed in PT several times before imaging. Staining for phospho-Smad1/5/8 [rbIgG Phospho-Smad1 (Ser463/465)/Smad5 (Ser463/465)/Smad8 (Ser426/428), Cell Signaling #9511] was carried out similar to that for dpErk, but with only one overnight incubation in primary antibody followed by an Alexa-based secondary antibody.

Embryos were mounted in gelatin and sectioned at 12 μm using a Leica CM1900 Cyostat. Sections were mounted with Permafluor (Thermo Scientific) with or without DAPI (10 $\mu\text{g/ml}$). Images after immunostaining or in situ hybridization were acquired using a SPOT SE camera and software using a Nikon Eclipse 80i microscope, and processed in Adobe Photoshop. DAPI color in Figs 4 and 5 is selectively lightened to increase visibility. Student's *t*-tests of statistical significance, assuming unequal variance, were performed, comparing treated and control embryos. *P* values are provided in supplementary material Table S1.

RESULTS

Inhibition of FGF signaling during gastrulation causes a medial expansion of lateral ectoderm markers and a loss of primitive streak markers

To evaluate the role of FGF signaling during gastrulation, we electroporated chick embryos at stage 3/3+/4- on the entire left side of the epiblast with a dominant-negative Fgfr1 (dnFgfr1) construct

cloned into a vector expressing nuclear-localized GFP (pCIG). Embryos were cultured for 16 hours, then assayed for the expression of lateral ectoderm markers *Msx1*, *Bmp4* and *Gata2*. *Msx1* and *Bmp4*, primarily expressed in the neural plate border (NPB) and in some non-neural ectoderm (NNE) tissue, are strongly expanded medially into the caudal neural plate (NP) and primitive streak upon FGF inhibition (*Msx1*, $n=8/12$, Fig. 1A; *Bmp4*, $n=7/8$, Fig. 1B). In addition, some of these embryos show a slight increase in the NPB domain (*Msx1*, $n=6/8$; *Bmp4*, $n=2/7$; not shown), whereas others are inhibited in the NPB (*Msx1*, $n=2/8$; *Bmp4*, $n=5/7$, Fig. 1B). *Gata2*, which marks NNE, is slightly expanded medially into the caudal NP after dnFgfr1 electroporation ($n=8/11$, Fig. 1C), but not to the extent that *Msx1* or *Bmp4* are. Control embryos electroporated with the empty vector pCIG display normal expression of *Msx1* ($n=11/12$, Fig. 1A'), *Bmp4* ($n=8/9$, Fig. 1B'), and *Gata2* ($n=12/12$, Fig. 1C'). These results suggest FGF signaling is required in the medial regions of the embryo to prevent the ectopic expansion of lateral markers, and might have a role in NPB development.

Next, we analyzed *Sox2*, a specifier of neural tissue. Inhibition of FGF/MAPK signaling before or during stage 3 is known to cause a loss of *Sox2* expression, whereas inhibition at stage 4 does not (Delfino-Machin et al., 2005; Stavridis et al., 2007; Wilson et al., 2000). Interestingly, *Sox2* expression is unaffected by electroporation of dnFgfr1 pCIG at stage 3+/4- when we see ectopic expression of lateral markers in the NP ($n=7/8$ normal, Fig. 1D). Earlier electroporation at stage 3 causes an inhibition of *Sox2* expression, as previously reported ($n=2/2$, not shown). Embryos electroporated with pCIG appear normal ($n=10/10$, Fig. 1D'). Known targets of FGF signaling, *Brachyury* (*Bra*) ($n=18/20$, Fig.

1E) and *Wnt8c* ($n=5/6$, Fig. 1F) are inhibited by dnFgfr1 electroporation, whereas the empty vector has no effect (*Bra*, $n=15/15$ normal, Fig. 1E'; *Wnt8c*, $n=3/3$ normal, Fig. 1F'). *Tbx6L*, a lateral mesoderm marker, is also inhibited by dnFgfr1 ($n=10/10$) whereas control embryos appear normal ($n=9/9$) (not shown). Statistical significance of $P<0.05$ was found in these experiments, and similar analysis was performed throughout our study and is provided as supplementary material Table S1. Taken together, these results demonstrate that FGF inhibition at gastrulation stages causes a medial shift in the expression of lateral ectoderm markers, and a loss of primitive streak and mesoderm markers.

FGF signaling is required for neural plate border development during gastrulation

Our analysis of *Msx1* and *Bmp4* suggests FGF signaling may have a role in NPB development, but we observe some inconsistencies in their responses to FGF inhibition, perhaps owing to slight variations in the stage of electroporation. Next, we performed a fine temporal analysis of FGF inhibition on Pax7, as it is expressed exclusively in the NPB at early stages and required for later NC development (Basch et al., 2006; Otto et al., 2006). The majority of embryos electroporated with dnFgfr1 at stage 3/3+ show reduced Pax7 expression ($n=13/23$, Fig. 2A). Interestingly, a smaller number of embryos electroporated at this stage ($n=6/23$) display a different phenotype altogether, presenting an ectopic expansion of Pax7 expression into the caudal NP accompanied by an increase in its endogenous neural fold domain anteriorly. If embryos are electroporated slightly later during gastrulation at stage 4-, most instead exhibit the expanded/increased phenotype ($n=9/11$, Fig. 2C,D). Some embryos electroporated at these stages

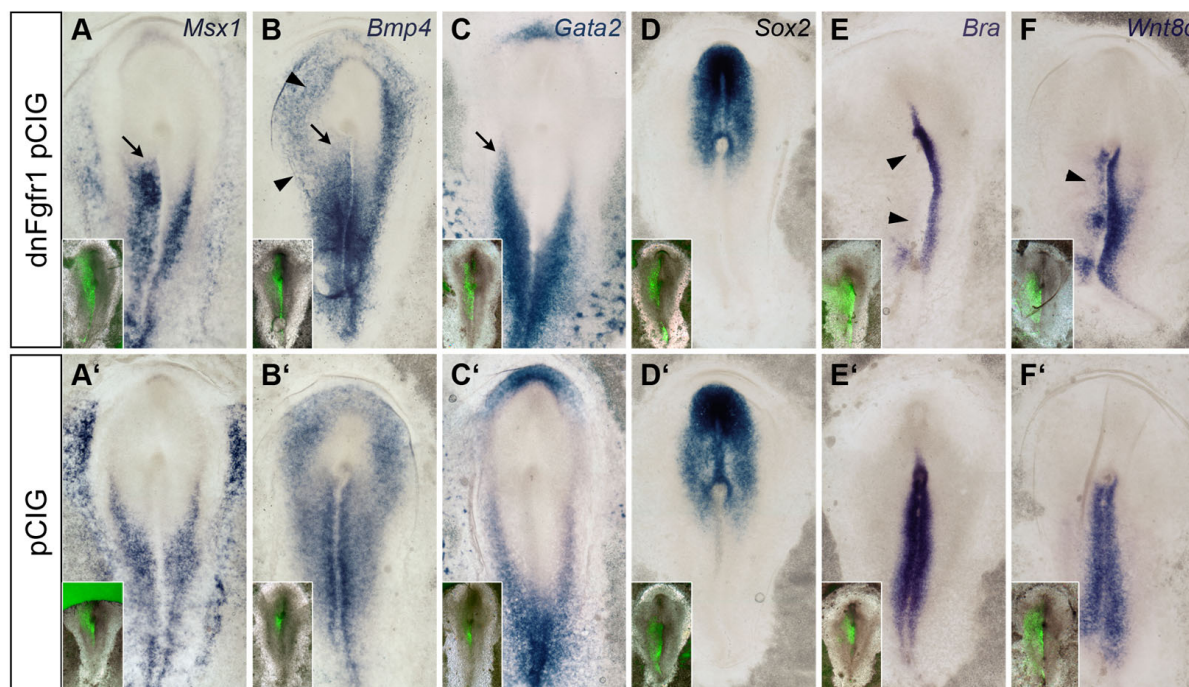


Fig. 1. FGF signaling is required to prevent ectopic expression of lateral ectoderm markers in medial tissues. (A-F) Inhibition of FGF signaling at gastrulation using a truncated Fgfr1 construct causes a strong medial expansion of NPB markers *Msx1* (A) and *Bmp4* (B), and a slight medial expansion of the NNE marker *Gata2* (C). The neural marker *Sox2* (D) is unaffected by electroporation at late gastrulation stages. FGF inhibition causes a complete or near-complete loss of the primitive streak markers *Bra* (E) and *Wnt8c* (F). (A'-F') Embryos electroporated with the empty vector control appear normal. Insets show electroporated tissues (green) after culture, before in situ hybridization. Arrows indicate ectopic expression. Arrowheads indicate inhibition.

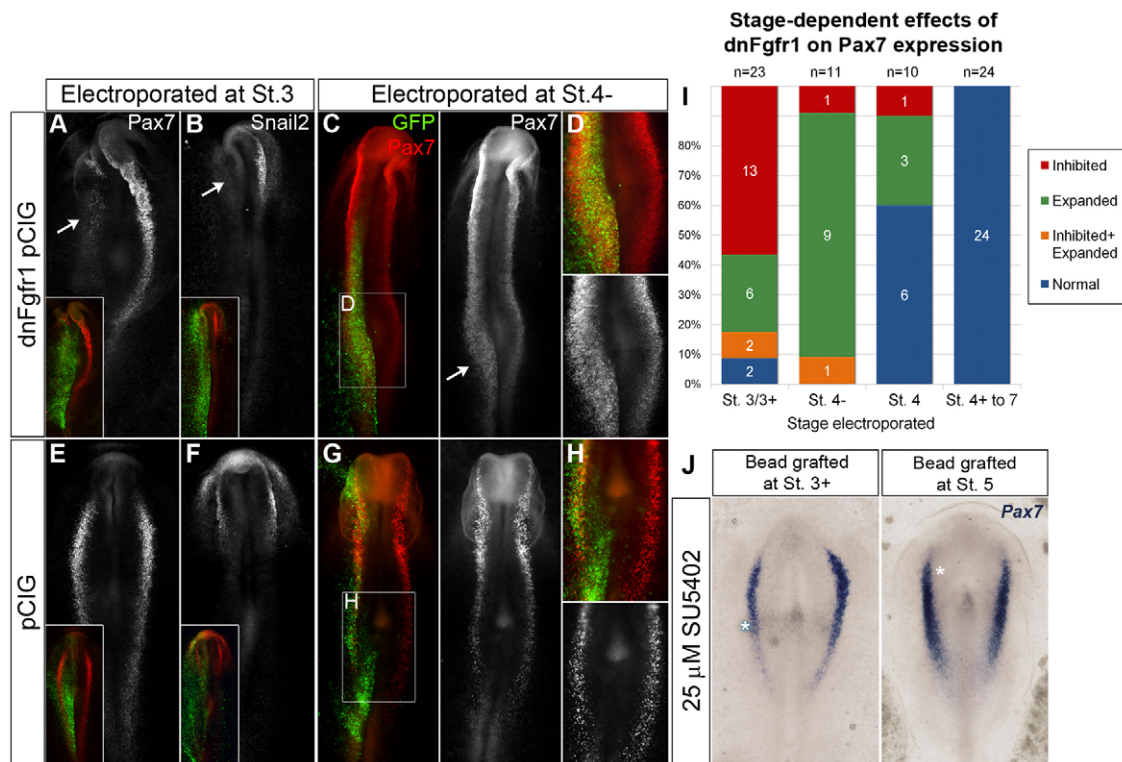


Fig. 2. FGF signaling is required during gastrulation for proper neural crest development. (A-H) Inhibition of FGF signaling during early gastrulation stages (stage 3/3+) causes an inhibition of Pax7 (A) and Snail2 (B) expression. Inhibition at late gastrulation stages (stage 4-) causes an ectopic expansion into the more medial regions of the embryo (C,D). Embryos electroporated with the empty vector control (E-H) appear normal. Insets in A,B,E,F show electroporated tissues (green). Arrows in A-C identify effects of FGF inhibition. **(I)** Distribution of dnFgfr1-induced effects on Pax7 expression, shown graphically. **(J)** Beads coated in 25 μ M SU5402 cause an inhibition of Pax7 when grafted at the pNPB at gastrula stage 3+, but cause no effect when grafted at the NPB at later stages. Embryos treated with beads coated in DMSO as a control display normal Pax7 expression (not shown). Asterisks indicate bead location.

show both an inhibition in the NPB/neural folds and expansion into the caudal NP ($n=2/23$ electroporated at stage 3/3+; $n=1/11$ at stage 4-). Electroporation of dnFgfr1 at the end of gastrulation (stage 4) results in mostly normal embryos ($n=6/10$), with a minority having abnormal phenotypes ($n=3/10$ expanded/increased, $n=1/10$ inhibited). Embryos electroporated after gastrulation all display normal Pax7 expression ($n=8/8$ electroporated at stage 4+, $n=9/9$ at stage 5, $n=5/5$ at stage 6 and $n=2/2$ at stage 7; data not shown). Stage-dependent effects of dnFgfr1 pCIG electroporation on Pax7 expression are summarized graphically in Fig. 2I. Embryos electroporated with the control vector exhibit normal Pax7 expression ($n=17/17$ electroporated at stage 3/3+, Fig. 2E, $n=8/8$ at stage 4-, Fig. 2G, $n=4/4$ at stage 4, $n=12/12$ if electroporated at stage 4+ or later, not shown).

To confirm that FGF signaling is required for Pax7 expression at the NPB, we grafted beads coated with SU5402, a chemical inhibitor of Fgfr transphosphorylation, or DMSO as a control during or after gastrulation (Fig. 2J). Beads were grafted between the prospective or proper NPB and the lower layers. We again saw Pax7 expression at the NPB was only sensitive to FGF inhibition during early stages of gastrulation (stage grafted, number inhibited: stage 3/3+, $n=7/11$; stage 4-, $n=1/5$; stage 4, $n=0/5$; stage 4+ to 7, $n=0/18$). Grafting of DMSO-coated beads yielded normal expression (stage 3/3+, $n=3/3$; stage 4, $n=4/4$; stage 4+ to stage 7, $n=11/12$). Taken together, these results suggest a delicate timing for FGF signaling in the early avian gastrula: the NPB appears to require FGF signaling until early gastrulation stages (3/3+), whereas the NP and primitive streak

remain sensitive to FGF inhibition until late gastrulation stages (4-4). In the stages following gastrulation, FGF signaling no longer contributes to Pax7 expression.

To determine whether FGF activity during gastrulation is necessary for later NC markers, we analyzed Snail2 expression. Most embryos electroporated with dnFgfr1 at stage 3/3+ present an inhibition of Snail2 expression ($n=5/9$, Fig. 2B), whereas fewer embryos show increased expression ($n=2/9$). Electroporation at stage 4-/4 causes a lower frequency of these effects ($n=2/8$ inhibited; $n=1/8$ increased), and any electroporation from stage 4+ until stage 8 has no effect ($n=0/13$, not shown). All control embryos appear normal ($n=8/8$ electroporated at stage 3/3+, Fig. 2E; $n=13/13$ at stage 4-/4; $n=3/3$ at stage 4+, 7, or 8). These results demonstrate that FGF signaling is essential up until gastrulation for NC induction in amniotes.

Early distributions of FGF receptor expression support a role for FGF signaling during gastrulation

Next, we examined the mRNA expression of the four FGF receptors at gastrula stages. *Fgfr1* is broadly expressed in much of the epiblast immediately preceding gastrulation at stage 2 (not shown), and by stage 3+ still encompasses the prospective NP (pNP) and prospective NPB (pNPB), but is excluded from the most lateral regions of the embryo that give rise to NNE (Fig. 3A,D). At later stages of gastrulation (stage 4-), *Fgfr1* is expressed most highly in the pNP, with lower levels found in the surrounding ectoderm (Fig. 3B), and

by stage 4/4+ continues to be most highly enriched in the NP (Fig. 3C). Throughout all early stages of development, *Fgfr1* is expressed at a low level in the primitive streak. *Fgfr2* and *Fgfr3* are not expressed strongly until stage 4, when they are found primarily in neural ectoderm and caudal NNE, respectively (supplementary material Fig. S2). *Fgfr4* is expressed strongly in much of the epiblast, as well as the node and the caudal primitive streak at gastrulation stages (Fig. 3E,F). By the end of gastrulation at stage 4/4+, the expression of *Fgfr4* has decreased and become more restricted (Fig. 3G). Combined, the spatiotemporal expression profiles of *Fgfr1* and *Fgfr4* demonstrate FGF signaling is capable of acting on the pNPB during gastrulation. Importantly, there is no FGF receptor expression in the mesoderm during gastrulation when FGF signaling is required for NC induction.

Erk activity in the early embryo depends on FGF signaling and is required for proper neural plate border development

As many recent studies demonstrate that FGFs act through the mitogen-activated protein kinase (MAPK) signaling pathway during embryogenesis, we examined the activation state of Erk1/2 proteins, the downstream effectors of MAPK signaling, using an antibody specific to the activated, dual-phosphorylated forms (abbreviated, dpErk). At stage 3+, dpErk is found in most nuclei throughout the gastrula, with the strongest levels found in the primitive streak and pNP (Fig. 3I). Sections demonstrate that epiblast cells within a given region display a wide variation in dpErk levels, but, overall, the medial epiblast contains a greater number of nuclei with high levels of dpErk when compared with the lateral epiblast (supplementary material Fig. S3A,D,E). Embryos stained in parallel without the primary antibody show no nuclear signal (supplementary material Fig. S3B,C). This demonstrates the presence of activated Erk1/2 proteins in the pNPB, and suggests a subtle gradient of MAPK activity in the epiblast during gastrulation.

Next, we electroporated stage 3+/4- embryos with dn*Fgfr1*, and then assayed dpErk levels. Embryos treated with dn*Fgfr1* show a strong inactivation of Erk1/2 proteins in all three tissue layers and the primitive streak ($n=9/9$, Fig. 4A,B), whereas embryos electroporated with the control vector display normal Erk activation ($n=7/7$ normal, Fig. 4C). We conclude that FGF signaling is responsible for the majority of Erk signaling in the early avian embryo.

To determine whether the MAPK signaling pathway is required for neural crest development, we overexpressed MAP kinase phosphatase 3 (Mkp3, also known as Dusp6), which dephosphorylates and inactivates Erk1/2, serving as a cell-autonomous inhibitor of Erk signaling. Embryos electroporated with Mkp3 pCIG display a strong inhibition of *Bra*, a direct target of Erk1/2 signaling ($n=9/9$, Fig. 4D), whereas embryos electroporated with pCIG appear normal ($n=8/8$, Fig. 4G). We then immunostained these embryos for Pax7 and GFP. In agreement with our dn*Fgfr1* electroporation results, Mkp3 overexpression also creates two different effects – inhibition of Pax7 in the domain of the neural folds and NPB, while also causing an ectopic medial expansion of Pax7 into the caudal NP and primitive streak ($n=5/5$, Fig. 4D'-F; control $n=0/5$, Fig. 4G').

NPB cells expressing high levels of GFP (high levels of Mkp3) express very little or no Pax7 compared with their neighboring cells (Fig. 4D',D'', arrowhead). In the caudal NP, we observed GFP-positive cells ectopically expressing Pax7, but also found that cells expressing the highest levels of GFP did not display ectopic Pax7

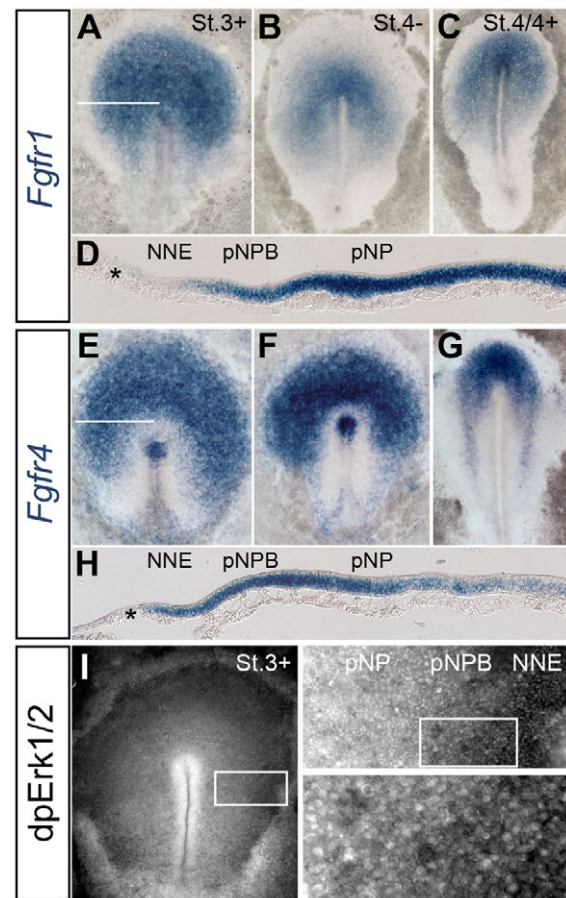


Fig. 3. FGF receptor expression and Erk1/2 activation during gastrula stages. (A-H) *Fgfr1* is expressed broadly in the epiblast during early gastrulation at stage 3+, including the pNPB (A), but later in gastrulation (stage 4-) becomes more restricted (B). By stage 4 at the end of gastrulation, *Fgfr1* is most highly expressed in the neural plate, with lower levels found in the surrounding tissues (C). *Fgfr4* is expressed in most of the epiblast during gastrulation, as well as in the node and caudal primitive streak (E,F). By stage 4, *Fgfr4* expression is reduced and more isolated to the anterior NP (G). Sections demonstrate that both *Fgfr1* (D) and *Fgfr4* (H) are expressed in the pNPB epiblast, but are not expressed in the mesoderm. The boundary between area opaca and area pellucida is marked with an asterisk. (I) Most epiblast cells contain activated Erk1/2 proteins in their nuclei, but the relative levels of dual-phosphorylated Erk1/2 (dpErk) vary between tissues. The primitive streak and pNP have the highest levels of dpErk, whereas nuclei of the pNPB contain an intermediate level of dpErk, when compared with the pNP and NNE.

expression. This implies that some level of Erk activity is necessary for cells to express Pax7. We additionally found cells that expressed little to no GFP that still displayed ectopic Pax7 expression, suggesting that there might be some non-cell-autonomous contributions to this phenotype.

FGF/MAPK signaling acts directly on the gastrula epiblast to specify the neural plate border and to prevent ectopic expression of lateral markers in the neural plate

Embryos electroporated broadly with FGF/Erk-inhibiting constructs during gastrulation stages exhibit an underdeveloped mesoderm (Fig. 4F, arrowhead), do not express the mesoderm

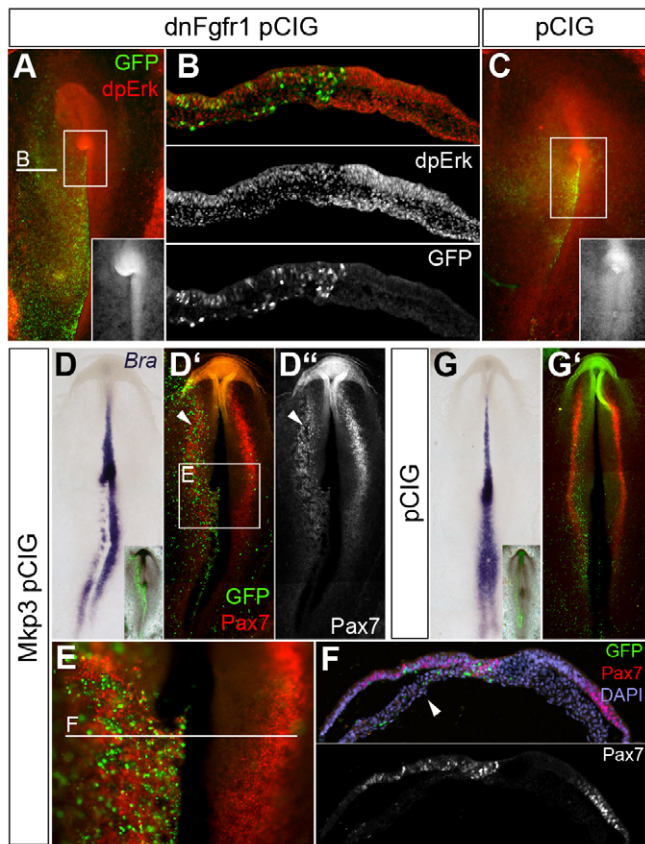


Fig. 4. FGF is responsible for Erk1/2 activation and Erk1/2 signaling is required for proper Pax7 expression.

(A–C) Electroporation of dnFgfr1 causes a strong inhibition of Erk1/2 activity (dpErk) in all tissue layers (B), while the empty vector control has no effect (C). Insets in A,C display high-power magnifications of dpErk staining – inhibition in A and normal expression in C. (D–E) Inhibition of Erk signaling by overexpression of Mkp3 causes an inhibition of *Bra* (D). In these embryos, Pax7 is inhibited in its endogenous domain (D', D'', arrowhead) and expanded medially (E). (F) Sections show expansion of Pax7 expression in the ectoderm layer and severe underdevelopment of the mesoderm on the electroporated side (arrowhead). (G, G') Embryos electroporated with the control vector have normal *Bra* and Pax7 expression. Insets in D, G show electroporated tissues after culture, before in situ hybridization.

markers *Bra* and *Tbx6L*, and display a curved primitive streak (see Fig. 1). As FGFs are involved in mesoderm development and mediating cell ingression through the primitive streak (Amaya et al., 1991; Amaya et al., 1993; Ciruna and Rossant, 2001; Ciruna et al., 1997; Hardy et al., 2011; Sun et al., 1999), some of the ectodermal changes might be secondary to the perturbation of mesodermal signals. To address this issue, we first tested the effects of FGF inhibition using an explant assay. Previous work from our lab has demonstrated that pNPB epiblast explants from stage 3 embryos cultured in isolation will go on to express Pax7 and form migratory NC cells and derivatives independently of mesoderm markers *Bra* and *Tbx6L* (Basch et al., 2006). Here, we dissected a strip of epiblast from the equator of the embryo (hypoblast removed) and cut it into ten equivalent-sized explants, excluding the primitive streak. Each of these pieces was then cut in half and cultured for 16 hours, with one half in media containing 10 μ M SU5402, and the other half in media containing DMSO. Explants

cultured in DMSO exhibited a normal distribution of Pax7 expression, with pNPB explants (2, 3, 8 and 9) having Pax7-positive nuclei, while more lateral (NNE, 1 and 10) and more medial explants (pNP, 4, 5, 6 and 7) displayed minimal or no Pax7 expression at all. By contrast, pNPB explants cultured in 10 μ M SU5402 do not express Pax7, whereas pNP explants 4, 5, 6 and 7 now ectopically express Pax7 ($n=8$, Fig. 5A). This supports the requirement of FGF signals in the pNPB epiblast to specify Pax7 expression, and in the pNP epiblast to prevent ectopic Pax7 expression.

To confirm whether FGF/MAPK signaling acts directly on the ectoderm, we restricted electroporation of Mkp3 pCIG to the pNPB at early stages of gastrulation (3/3+) or to the pNP at late gastrulation (4–). This results in expression of our construct exclusively in the ectoderm of the embryo, without adverse effects on mesoderm ingression. Mkp3 overexpressed in prospective NC cells caused a cell-autonomous loss of Pax7 expression in its endogenous domain ($n=8/8$, Fig. 5B–E; control $n=0/6$, not shown), whereas electroporation in the pNP generates ectopic Pax7 expression in the NP ($n=6/7$; control $n=0/4$; supplementary material Fig. S4). Similarly, NP-restricted electroporation of dnFgfr1 generates ectopic expression of Pax7 ($n=8/8$, Fig. 5F,G), *Msx-1* ($n=6/9$, not shown) and *Bmp4* ($n=4/7$, not shown), while control electroporation has no effect (Pax7 $n=0/7$, *Msx-1* $n=0/9$, *Bmp4* $n=1/9$, not shown). Some of these embryos display restricted electroporation to the anterior NP (and not to the posterior NP), and present cell-autonomous ectopic expression of lateral markers, demonstrating that FGF/MAPK inhibitory effects are not limited to the caudal NP. Henceforth, we refer to NP effects in general, without axial quality. These results, together with explant experiments (Fig. 5A), demonstrate that FGF/MAPK signaling acts directly on the gastrula epiblast to both specify the NPB and prevent the ectopic expression of NPB markers in the NP.

FGF/MAPK signaling is known to be necessary for the expression of the neural specifiers *Sox2* and *Sox3* up until gastrulation (Delfino-Machin et al., 2005; Stavridis et al., 2007; Wilson et al., 2000). Our results complement these studies, but suggest that *Sox2* is insensitive to FGF inhibition at late gastrulation stages (Fig. 1D). In order to determine whether the ectopic expression of NPB markers in the NP is accompanied by the loss of the neural tissue, we electroporated embryos with dnFgfr1 pCIG in the pNP at stage 3+/4–, then co-stained for Pax7 and an antibody that recognizes both *Sox2* and *Sox3*. Surprisingly, we found that neural specification is unaffected by inhibition of FGF signaling at late gastrulation stages as indicated by normal *Sox2/3* expression, though cells fated to become NP do indeed express Pax7 ($n=5/5$; control, $n=0/6$; supplementary material Fig. S5). These findings demonstrate a narrow window during gastrulation when the NP has been specified, but still remains sensitive to inhibition of FGF signaling.

Smad1/5/8 signaling becomes active after gastrulation and is required for proper neural plate border development

Several reports have implicated BMP signaling in avian NPB formation and NC development (Basch et al., 2006; Liem et al., 1997; Liem et al., 1995; Linker et al., 2009; Patthey et al., 2009; Patthey et al., 2008; Selleck et al., 1998; Streit and Stern, 1999), and one study monitored BMP/Smad signaling (Faure et al., 2002), but Smad activation has not been directly related to early neural crest development. We demonstrated that Pax7 and *Bmp4* have responded similarly to FGF inhibition, and wondered to what

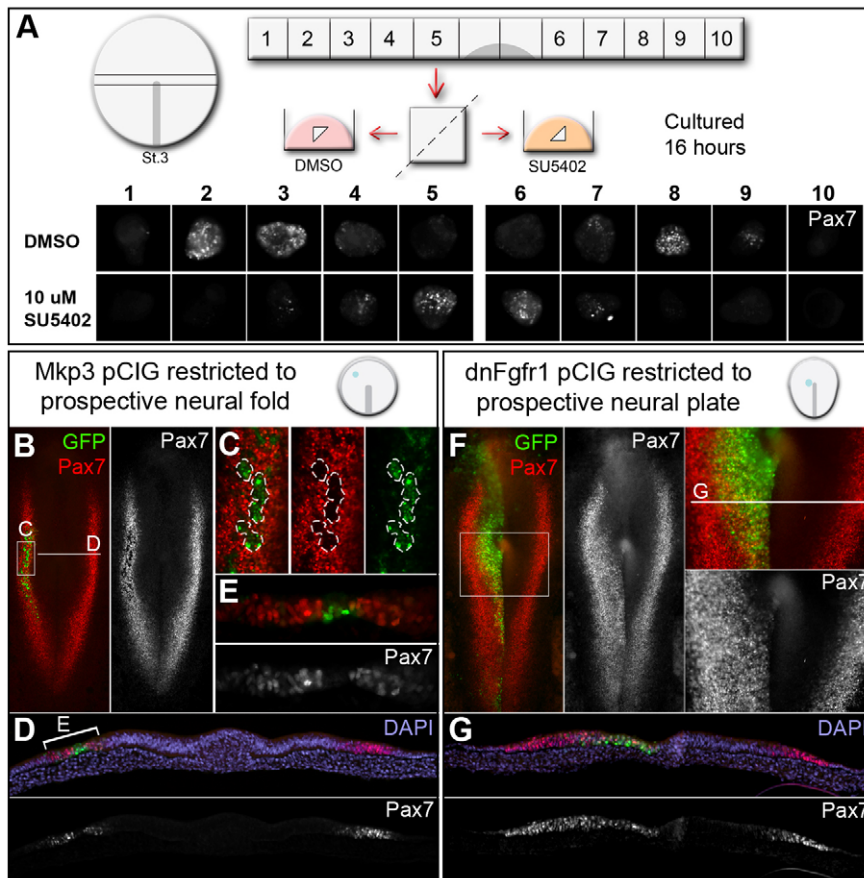


Fig. 5. FGF acts on the epiblast, independently of other tissues, to elicit changes in the ectoderm. (A) A strip of epiblast tissue was taken from the center of a stage 3 embryo, then cut into 10 pieces, excluding the primitive streak. Each piece was cut in half and cultured with SU5402 or DMSO as a control. SU5402 causes a loss of Pax7 from explants that normally express it, and an ectopic upregulation in explants fated to become neural tissue. **(B-E)** Restricting Mkp3 pCIG to the prospective neural fold at early stages of gastrulation (stage 3/3+) causes a cell-autonomous loss of Pax7. **(F,G)** Restricting dnFgfr1 pCIG to the pNP at late stages of gastrulation (stage 4+) elicits ectopic expression of Pax7 in medial tissues.

extent BMP/Smad signaling might regulate Pax7 expression at early developmental stages. As a first step, we analyzed the distribution of phosphorylated Smad1/5/8 (pSmad1) and Pax7 expression from gastrulation to neurulation (Fig. 6A-I). Interestingly, pSmad1 is barely detectable at early gastrulation stages (Fig. 6A, $n=6$) and first becomes apparent in the posterior territory of the embryo at late gastrulation stages (Fig. 6B, $n=5$), suggesting there is very little or no BMP activity during gastrulation (also see supplementary material Fig. S6). By stage 4/4+, the caudal primitive streak and caudal ectoderm begin to display high levels of pSmad1 ($n=8$). By early stage 5, pSmad1 is found in much of the NNE and primitive streak, as well as in NPB cells that also express Pax7, but is noticeably absent from the NP (Fig. 6D, $n=11$). Although many Pax7-expressing cells have a strong nuclear enrichment of pSmad1 (Fig. 6E, closed arrowheads), some cells with robust Pax7 expression have low levels of pSmad1 (Fig. 6E, open arrowheads). Still, all cells that express Pax7 have detectable levels of pSmad1, greater than that observed in the NP.

At stage 7, the NNE, primitive streak and NPB continue to express high levels of pSmad1, as do the anterior neural folds (Fig. 6F-I). Additionally, the NP has a low pSmad1 level at this stage, with slightly more activity in the caudal NP (Fig. 6F). Sections suggest that the neural fold/NPB, marked by Pax7 (Fig. 6H, arrowheads), has an overall intermediate level of Smad1/5/8 activity when compared with that seen in the NNE and the NP. However, cells that express Pax7 appear in a territory of higher Smad1/5/8 activity (Fig. 6I, bounded by dotted lines) when compared with cells immediately adjacent that do not express Pax7. Embryos processed in parallel without incubation in primary antibody show no nuclear signal (supplementary material Fig. S6).

This analysis suggests that Smad signaling plays a role in NPB formation and maintenance, although it is likely to be required after gastrulation.

To directly assay whether signaling through Smad1/5/8 proteins is required for NPB formation, we overexpressed Smad6, an intracellular inhibitor of Smad1/5/8 signaling, using a vector (pCAB) that also expresses non-localized GFP. Electroporation of Smad6 pCAB at stage 3+/4- causes a strong decrease in pSmad1 levels ($n=4/4$; control, $n=0/3$; supplementary material Fig. S7A,D) and a complete loss of *Gata2* ($n=3/3$; control $n=0/3$; supplementary material Fig. S7B,E), a direct target of BMP/Smad signaling.

We then analyzed the NPB markers *Msx1* and Pax7 in response to Smad6 overexpression. Widespread electroporation of Smad6 pCAB causes a reduction and overall disorganization of *Msx1* ($n=3/3$; supplementary material Fig. S7C) and Pax7 ($n=7/7$, Fig. 7A,A'). With Pax7 being expanded laterally into the NNE along the entire rostrocaudal axis. These embryos also display a similar lateral expansion of Sox2/3, though expression in the NP appears unaltered ($n=7/7$, Fig. 7A,A"). Embryos electroporated with the control vector appear normal (*Msx1*, $n=3/3$, supplementary material Fig. S7F; Pax7, $n=5/5$, Fig. 7B,B'; Sox2/3, $n=5/5$, Fig. 7B,B"). We then restricted Smad6 electroporation to the pNPB and analyzed Pax7 expression. NPB cells that express high levels of Smad6 show an inhibition of Pax7 expression when compared with adjacent cells that express little or no Smad6 ($n=5/6$, Fig. 7C). Control embryos appear normal ($n=4/4$, not shown). These results support a role for BMP/Smad signaling in the formation and proper positioning of the NPB, and demonstrate that loss of Smad signaling in the ectoderm causes a lateral expansion of more medial cell fates, at the expense of NNE.

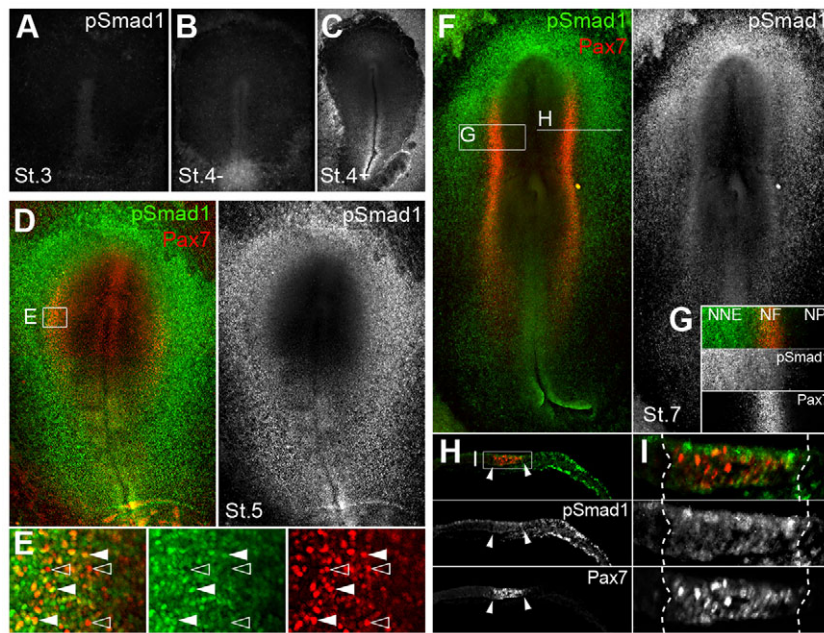


Fig. 6. Smad1/5/8 signaling is undetectable during gastrulation, but activated at the neurula stage. (A-E) Activated Smad1/5/8 (pSmad1) is barely detectable in gastrulation-stage embryos (A,B), but is progressively activated in caudal tissues as gastrulation ends. By stage 4+, strong pSmad1 is found in the caudal primitive streak and NNE (C). At the early neurula stage (stage 5), pSmad1 is observed in the NNE, and in the NPB (D) in cells that express Pax7 (E, closed arrowheads). Cells also expressing low levels of pSmad1 are seen to express Pax7 (E, open arrowheads). (F-H) At stage 7, strong pSmad1 activation is found in the NNE and throughout the neural folds and NPB (F) in cells that express Pax7 (G). The neural folds, expressing Pax7 (H, region between arrowheads) have an intermediate level of pSmad1 when compared with the NNE and NP. (I) Cells expressing Pax7 (I, bounded by dotted lines) have higher levels of pSmad1 when compared with adjacent cells that do not express Pax7 (I, outside dotted lines). NF, neural fold.

Ectopic Pax7 expression in the neural plate depends on attenuated FGF signaling and active Smad1/5/8 signaling

As Smad1/5/8 signaling is required for the proper expression of Pax7, and because loss of FGF signaling in the NP causes an ectopic expression of both *Pax7* and *Bmp4* in that domain, we argued that ectopic Pax7 expression in the NP might be a consequence of medially expanded Smad1/5/8 signaling. To determine whether ectopic *Pax7* expression was dependent on Smad signaling, we inhibited both FGF and Smad signaling in the pNP by co-electroporating dnFgfr1 pCIG and Smad6 pCA β at late gastrulation stages 3+/4-. Electroporation of both empty vectors into the pNP caused no effect on Pax7 expression ($n=9/9$ normal, Fig. 8A), as did electroporation of Smad6 pCA β + pCIG ($n=4/4$

normal, Fig. 8B). As expected, electroporation of dnFgfr1 pCIG + pCA β generated ectopic Pax7 expression in the NP ($n=6/8$, Fig. 8C). Significantly, co-electroporation of both inhibitory vectors dnFgfr1 pCIG + Smad6 pCA β did not yield ectopic Pax7 expression ($n=6/8$ normal, Fig. 8D), demonstrating that Smad1/5/8 signaling is required for Pax7 to be ectopically induced in the NP. These results demonstrate Smad1/5/8 signaling is necessary for ectopic Pax7 expression when FGF signaling is inhibited in the prospective NP.

DISCUSSION

FGF/MAPK signaling and the tissues involved in avian neural crest induction

We have characterized the participation of the FGF/MAPK signaling pathway in early NC induction during gastrulation and unveiled multiple roles in the development of ectodermal tissues. Using the chick model, our work constitutes the first major effort to analyze the participation of FGF signaling in NC induction in amniotes. Recent evidence from *Xenopus* suggests that FGF induces the NC indirectly, by activating *Wnt8* expression in the lateral mesoderm (Hong et al., 2008). By contrast, our results demonstrate a novel requirement for FGF signaling within the pNPB epiblast of the gastrula, independent of mesodermal signals (Fig. 5). Multiple studies from *Xenopus* have demonstrated the capacity of the ectoderm to respond to exogenous FGF signals in the absence of mesodermal tissue, producing strong induction of NC markers (Bang et al., 1997; Hong et al., 2008; LaBonne and Bronner-Fraser, 1998; Mayor et al., 1995; Monsoro-Burq et al., 2003; Villanueva et al., 2002). Two of these studies also show that FGF signaling is necessary for NC markers in BMP-compromised ectoderm when conjugated with mesoderm (Monsoro-Burq et al., 2003; Villanueva et al., 2002). However, FGF has not yet been shown to act on the prospective NC in intact *Xenopus* embryos.

Yet our results do not directly refute any additional input from mesodermal signaling in the amniote gastrula. The mesoderm underlying the pNPB lacks FGF receptor expression during gastrulation (Fig. 3D,H), suggesting it does not receive FGF signals during the first step of NC induction in the chick. FGFs are crucial

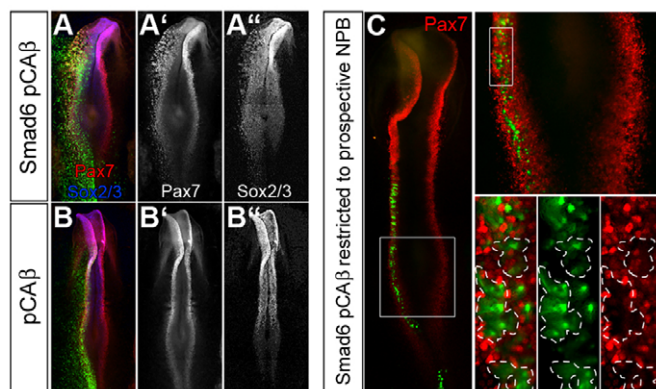


Fig. 7. Smad1/5/8 signaling prevents lateral expansion of neural and NPB markers and is required for Pax7 expression in the NPB. (A-A'') Broad electroporation of Smad6 causes a lateral expansion of Pax7 (A') and Sox2/3 (A'') into the NNE. Pax7 is inhibited in the neural fold, but Sox2 expression in the NP appears normal. (B-B'') Electroporation of the empty vector produces normal expression of Pax7 (B') and Sox2/3 (B''). (C) Cells in the NPB expressing high levels of Smad6 show an inhibition or complete loss of Pax7 when compared with adjacent cells. GFP (green) marks the location of the vectors.

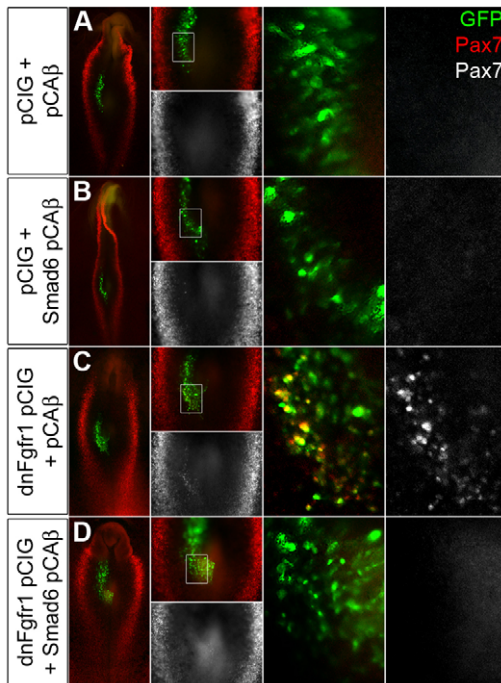


Fig. 8. Ectopic Pax7 expression in the NP depends on attenuated FGF signaling and active Smad1/5/8 signaling.

(A–D) Electroporation of the pNP with empty control vectors pCIG and pCAβ (A) or inhibition of Smad1/5/8 signaling using Smad6 pCAβ+pCIG (B) causes no change in Pax7 expression. Inhibition of FGF signaling using dnFgfr1 pCIG+pCAβ causes an ectopic increase in Pax7 expression (C), but co-electroporation of both FGF-inhibiting and Smad1/5/8-inhibiting vectors prevents ectopic Pax7 expression (D).

for mesoderm formation (Hardy et al., 2011), however, and thus could be considered to be responsible for mesodermal signaling. Although the mesoderm has not yet been implicated in amniote NPB formation, at later stages of development, both mesoderm and ectoderm are capable of inducing the NC in chicks (Selleck and Bronner-Fraser, 1995).

In *Xenopus*, the current model of early NC development proposes an initial inductive step during gastrulation that is dependent on Wnt activation and BMP inhibition, followed by a maintenance step during neurulation where both BMP and Wnt signaling are active (Steventon et al., 2009). This two-phase model is supported by chick explant studies (Patthey et al., 2009), but in vivo confirmation is lacking. Our analysis of the spatiotemporal distribution of Smad1/5/8 activation fits with the model; we find minimal Smad1/5/8 activity during gastrulation when FGF/MAPK signaling acts on much of the epiblast, and then a robust Smad1/5/8 activation colocalizing with Pax7 during neurulation (Fig. 6) when most of the FGF/MAPK activity is restricted to the neural plate (Fig. 3) (Lunn et al., 2007). We note that FGF may participate in BMP-antagonism in the pNPB during gastrulation, allowing the inductive step to proceed. We also demonstrate the requirement for Smad1/5/8 signaling in the NPB itself (Fig. 7C), supporting previous evidence for a late phase of BMP signaling (Garcia-Castro et al., 2002; Liem et al., 1995; Patthey et al., 2009; Patthey et al., 2008; Selleck et al., 1998).

Recent reports using epiblast explants (Patthey et al., 2009; Patthey et al., 2008) and early embryos (Basch et al., 2006) demonstrate that Wnt signaling is required from the earliest stages

of gastrulation, but the identity of the Wnt ligand(s) responsible for the inductive signal in amniotes remains unknown. In addition to acting directly on the pNPB, FGF/MAPK signaling also contributes to Wnt ligand expression in the primitive streak, positively regulating *Wnt3a*, *Wnt5b* and *Wnt8c* in the chick (Fig. 1F) (Hardy et al., 2011; Olivera-Martinez and Storey, 2007). Although this places *Wnt5b* and *Wnt8c* expression far from the pNPB at gastrula stages, their participation in amniote NC induction has not been tested. Alternatively, *Wnt1*, *Wnt2b*, *Wnt3a*, *Wnt6* and *Wnt7a* are expressed in the gastrula epiblast (S. Chapman, personal communication) (<http://geisha.arizona.edu/geisha>), making these potential candidates to be targeted by FGF/MAPK signaling. Interestingly, experiments at later stages have implicated Wnt6 in the regulation of Pax7 and later NC development (Garcia-Castro et al., 2002; Otto et al., 2006; Schmidt et al., 2007). It will be exciting to determine whether FGF signaling contributes to Wnt activity in the pNPB epiblast.

Implications for neural induction and early signaling in neural crest induction

In the chick, studies on neural induction point to a direct requirement for FGF signaling within the epiblast from pre-gastrula stages (Streit et al., 2000; Wilson et al., 2000; Wilson et al., 2001). More recently, Erk1/2 signaling was shown to be necessary for neural induction up until gastrulation (Stavridis et al., 2007). Together, these studies suggest that FGF, via the Erk signaling cascade, acts on the medial epiblast from the blastula through to the gastrula stage to specify neural tissue. Our results suggest a similar requirement for FGF/MAPK signaling within the pNPB epiblast, with a crucial point during gastrulation when FGF activities cease to affect the endogenous domain of Pax7. This lends support to a potential shared mechanism of neural and NC specification, orchestrated by FGF/MAPK signaling. However, in sharp contrast to promoting the expression of NPB makers in the pNPB epiblast, we find FGF/MAPK signaling is required in the pNP instead to prevent the expression of these same markers, independently of its role in neural specification. We note that some effects of FGF/MAPK inhibition in the NP are found anterior to the node, whereas other electroporations also encompass the caudal NP, a region capable of yielding mesodermal and neural derivatives termed the ‘stem zone’ (Delfino-Machin et al., 2005). As lateral ectoderm markers can be induced in the NP for a short period after FGF signaling is necessary for neural and NPB specification, these activities may be separable. Thus, FGF/MAPK signaling may act similarly on both the pNP and pNPB early on, but inhibition of FGF signals has different outcomes in the two regions at the gastrula stage.

It has been proposed that cells respond to varying levels of MAPK signaling, such that very high levels specify mesoderm tissue, and lower levels are needed to allow the neural program to proceed (Delaune et al., 2005). At gastrula stages, we see a modest difference in levels of activated Erk1/2 proteins in nuclei of lateral versus medial epiblast (Fig. 3; supplementary material Fig. S3), suggesting the low level in the lateral epiblast might specify the NPB, while progressively higher levels promote the generation of neural and mesodermal tissue. As Mkp3 overexpression causes a cell-autonomous loss of Pax7, we reason that some level of Erk1/2 signaling in the pNPB is required for Pax7 expression. In support, Pax7 is not ectopically induced in NP cells expressing extremely high levels of Mkp3 (cells that have the highest level of Erk inhibition). Still, the difference in Erk activation between nuclei is subtle, and may not be significant enough to elicit differential gene expression.

Although NP and NPB markers require FGF/MAPK signaling up until gastrula stages, the progenitors of these populations appear to be specified earlier. Explant studies that isolate the pNP and pNPB epiblast demonstrate that neural tissue is specified during or before the blastula stage in the chick (Wilson et al., 2000; Wilson et al., 2001), whereas the NC seems to be specified just before gastrulation (Patthey et al., 2009). These findings suggest that cells of the pNP and pNPB are inherently different by the gastrula stage, and probably respond distinctly to FGF inhibition due to their different intracellular characteristics. What caused these differences? Our study, together with other recent findings, suggests prospective neural and NC populations both require BMP inhibition and active FGF/MAPK signaling, but they differ in the status of canonical Wnt signaling (Patthey et al., 2009; Wilson et al., 2000; Wilson et al., 2001). Interestingly, nuclear-localized β -catenin and Wnt ligands are found in the lateral epiblast, but are absent from the medial, neural-specified epiblast at blastula stages (Roeser et al., 1999; Skromne and Stern, 2001), prompting an analysis of the intrinsic differences in the epiblast at this stage.

The participation of FGF/MAPK signaling machinery

What molecules in the FGF signaling machinery are likely to participate in NC induction? Our expression data demonstrate that both *Fgfr1* and *Fgfr4* could potentially serve as receptors for prospective neural or NC tissue during gastrulation (Fig. 3). Several canonical FGF ligands are expressed during chick gastrula stages, including *Fgf3*, *Fgf4*, *Fgf8* and *Fgf18* (Karabagli et al., 2002; Wilson et al., 2000; Wilson et al., 2001). Of these, *Fgf3* and *Fgf8* have been implicated in neural induction and could also mediate FGF effects on the pNPB. *Fgf3* is expressed in the epiblast from pre-gastrula stages (Wilson et al., 2000; Wilson et al., 2001), whereas *Fgf8* is expressed in the hypoblast as well as the primitive streak and newly ingressing mesendoderm (Karabagli et al., 2002; Streit et al., 2000). In addition to its regulation of *Wnt8* expression in *Xenopus* NC induction (Hong et al., 2008; Hong and Saint-Jeannet, 2007; Monsoro-Burq et al., 2005), *Fgf8* has been shown to induce *Msx1* (Streit and Stern, 1999) and many neural markers (Albazerchi and Stern, 2007) in the chick.

MAPK signaling is known to regulate relevant patterning genes such as Wnt ligands and BMP antagonists (Branney et al., 2009; Hardy et al., 2011), but the mechanism of MAPK action has yet to be determined in this context. Interestingly, Erk1/2 can phosphorylate the linker domain of Smad1/5/8 proteins, promoting polyubiquitylation by Smurf1 and subsequent degradation of the Smad, thereby inhibiting the pathway cell-autonomously (Fuentelba et al., 2007; Kretschmar et al., 1997; Sapkota et al., 2007). This pathway is crucial for *Xenopus* neural development (Fuentelba et al., 2007; Pera et al., 2003), and may also operate in the lateral avian epiblast fated to become NC cells. We have shown that inhibition of FGF/MAPK signaling in the pNP causes ectopic upregulation of Pax7, cell-autonomously (Fig. 5), and that this upregulation requires Smad signaling (Fig. 8). Potentially, the inhibition of FGF/MAPK signaling alleviates the phosphorylation of the Smad linker domain, resulting in a higher level of Smad signaling in the forming NP and invoking the expression of Smad1/5/8 targets without modifying C-terminal phosphorylation. This mechanism could account for the inability of Noggin (a secreted BMP antagonist) to eliminate lateral markers from medial epiblast explants treated with an FGF inhibitor (Wilson et al., 2001), and argues that FGF-

mediated MAPK phosphorylation of the Smad1/5/8 linker domain is a fundamental mechanism to promote neural development. An attractive hypothesis is that MAPK-dependent linker phosphorylation could also underlie the absence of Smad1/5/8 activity in the epiblast during gastrulation. Thus, the widespread distribution of Erk1/2 activity during gastrulation would explain the lack of observed Smad1/5/8 activation until neurulation, when ectodermal Erk1/2 activity is then restricted to the NP.

FGF signaling in *Xenopus* and zebrafish is also known to contribute to the expression of *Chordin* and *Noggin*, secreted BMP inhibitors (Branney et al., 2009; Fletcher and Harland, 2008; Kudoh et al., 2004). If we suppose FGF in the chick is also responsible for the activation of Wnt signaling during the gastrula stage of NC induction, it is possible that the proper level of FGF/MAPK activity could be sufficient to induce the initial NC program (Fig. 9). However, GSK3 was also shown to phosphorylate the Smad1 linker and contribute to the degradation pathway, though it first requires MAPK phosphorylation to 'prime' the linker domain. The same study also demonstrated that canonical Wnt signaling can prolong the period of Smad1 activity by inhibiting GSK3 and preventing or slowing degradation of Smad1 (Fuentelba et al., 2007). Thus, if FGF activates Wnt signaling, it would counterbalance MAPK inhibition of Smad signaling. The real challenge in the years to come is to determine which mechanisms of crossregulation are relevant to NC induction and how they integrate to activate the key regulatory transcription factors. In conclusion, our results illustrate the complex time-dependent roles of FGF/MAPK signaling in the early ectodermal development of amniotes and provide a new perspective into its role in NC induction.

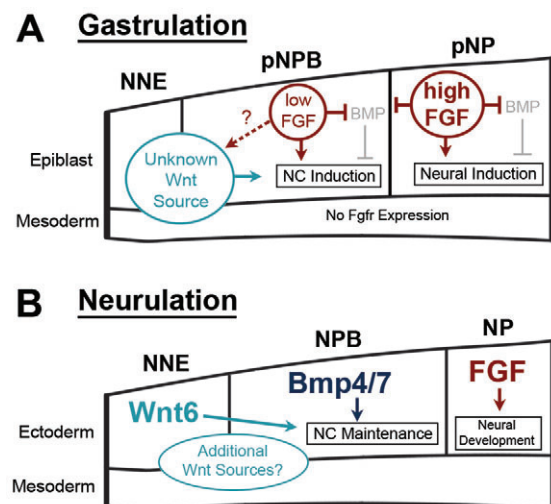


Fig. 9. Proposed mechanism of FGF participation in early neural crest development in avians. (A) FGF and Wnt activity both contribute to neural crest induction at gastrula stages in the pNPB epiblast. It is not yet clear whether FGF activates the Wnt signal responsible for induction, but FGF probably contributes to BMP inhibition on multiple levels. The source of the Wnt signal has not yet been identified. (B) During neurulation, FGF is no longer required in NC cells, but is active in further neural development. Wnt and BMP signaling cooperate to maintain the NC in the NPB. Wnt6 in the NNE and Bmp4/7 in the NPB are probably involved, although other molecules may also participate.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

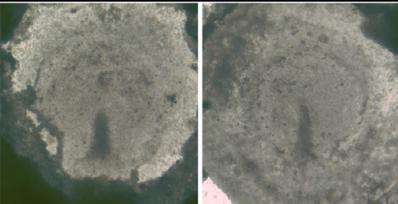
Supplementary material available online at
<http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.070276/-DC1>

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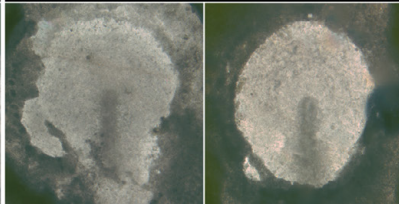
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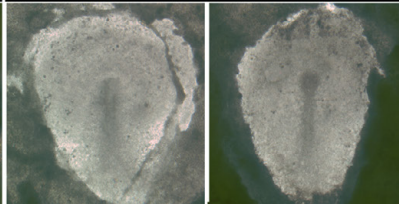
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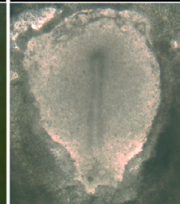
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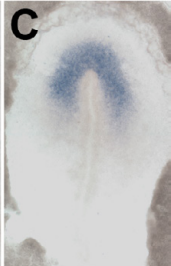
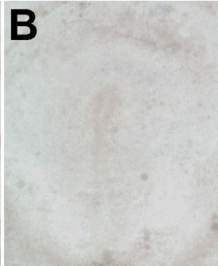
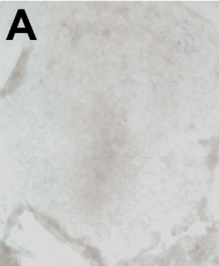
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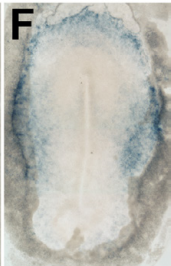
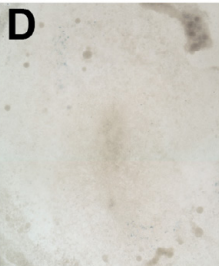
Stage 4



Fgfr2

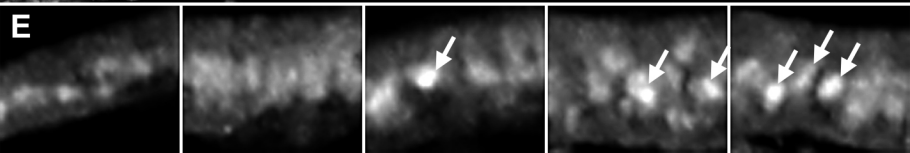
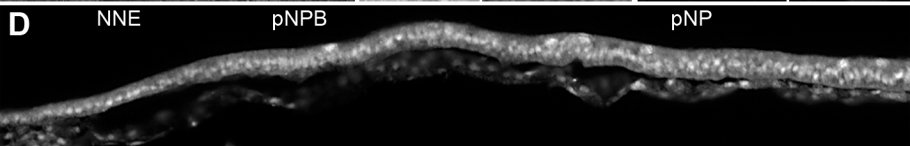
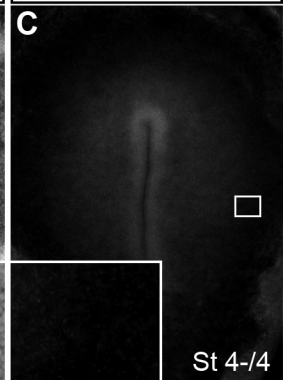
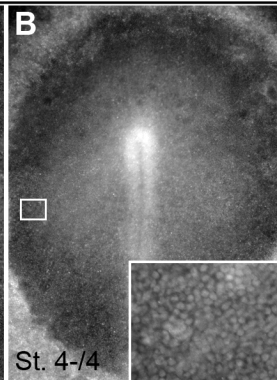
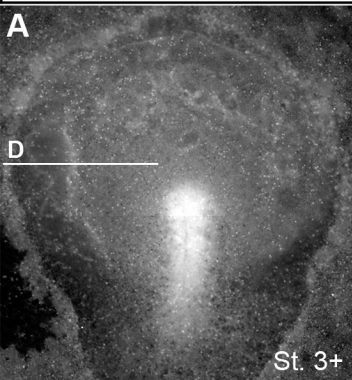


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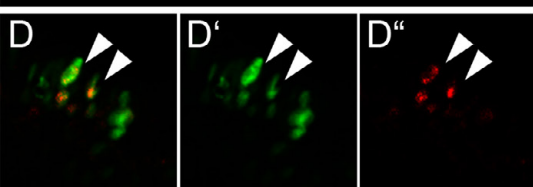
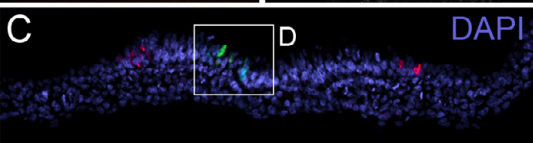
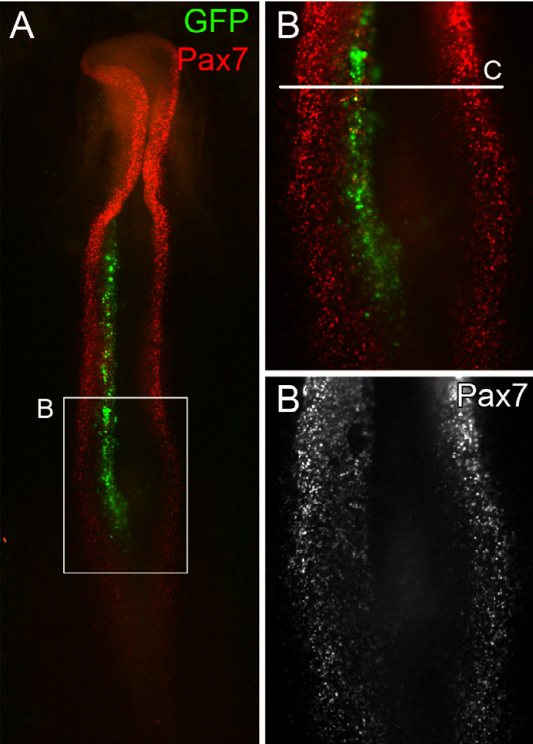


dpErk1/2

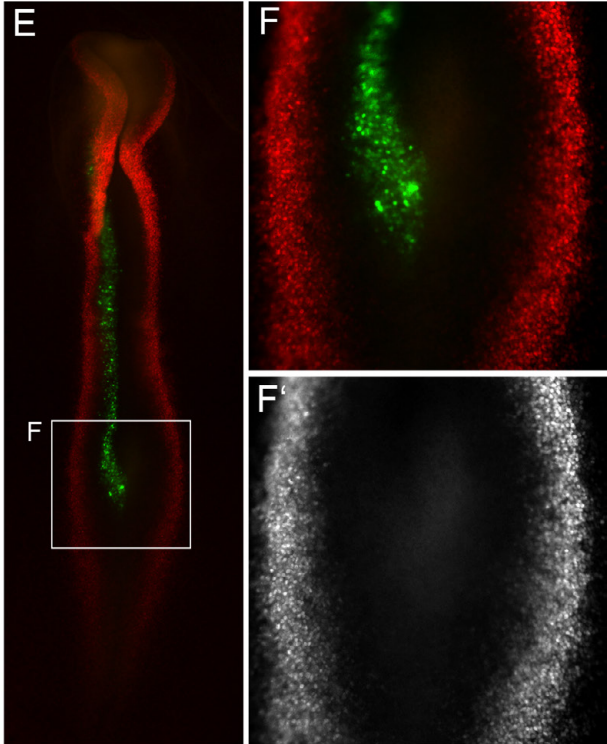
No 1° antibody

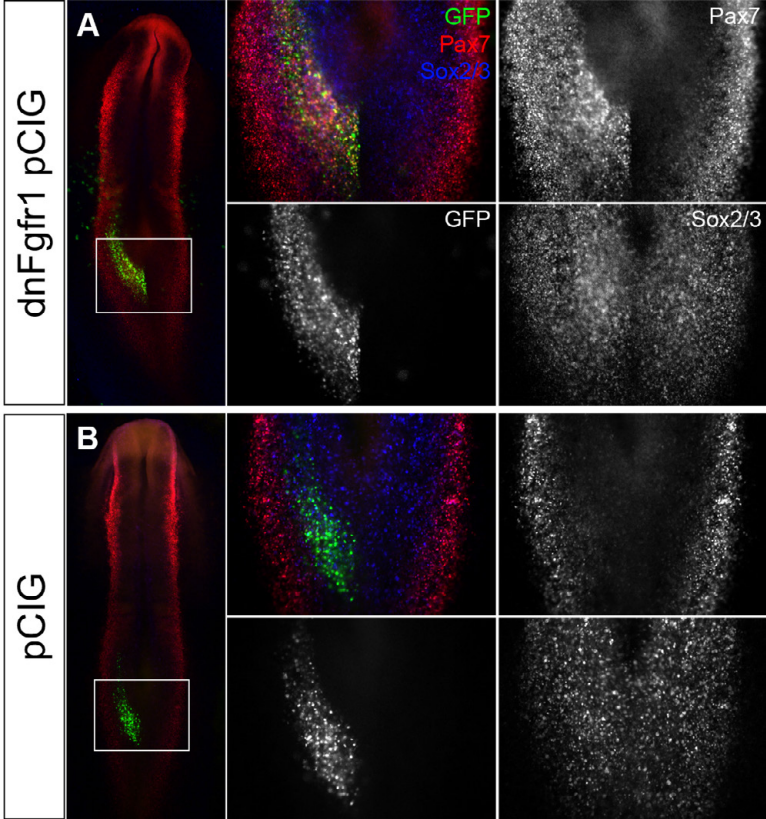


Mkp3 pCIG



pCIG

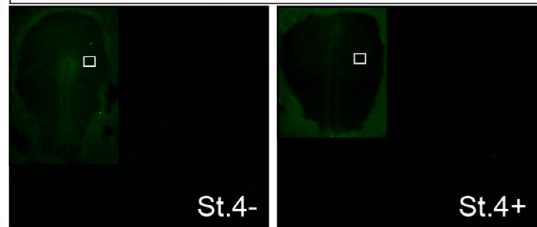
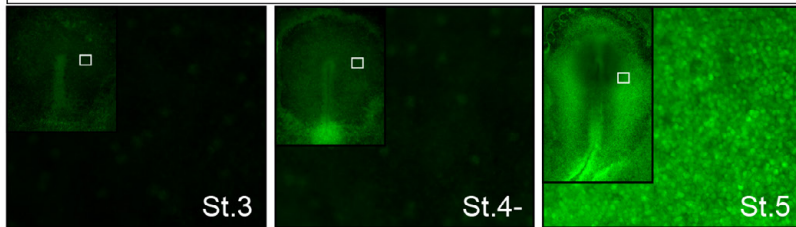




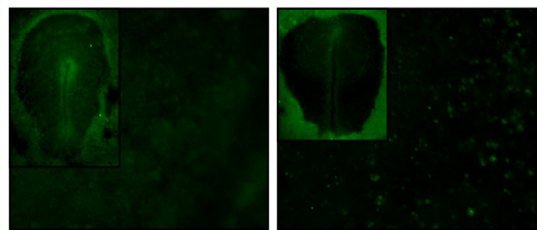
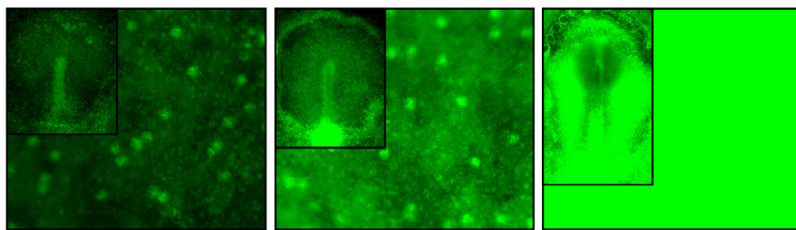
pSmad1

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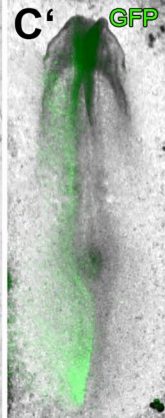
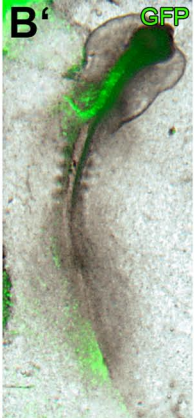
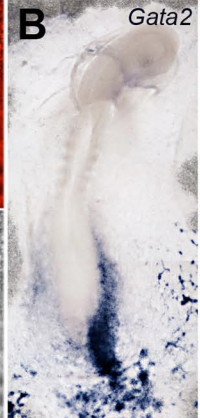
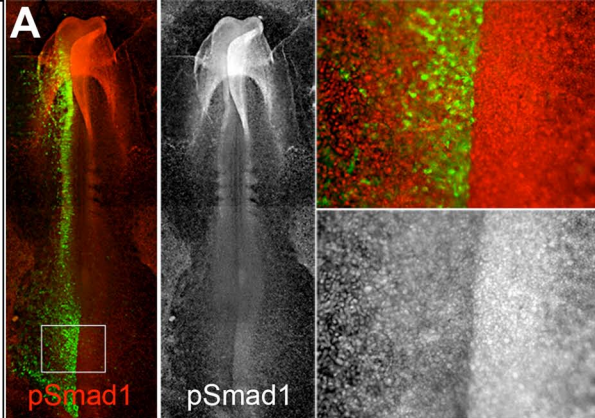
A



B



Smad6 pCA β



pCA β

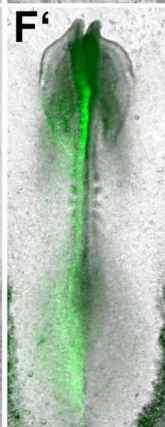
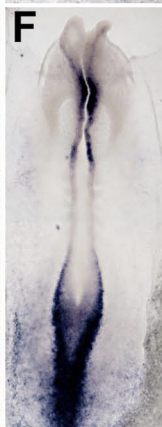
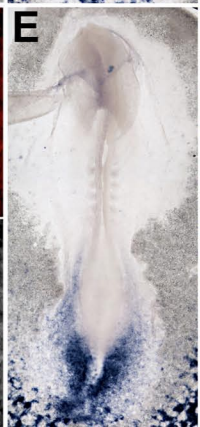
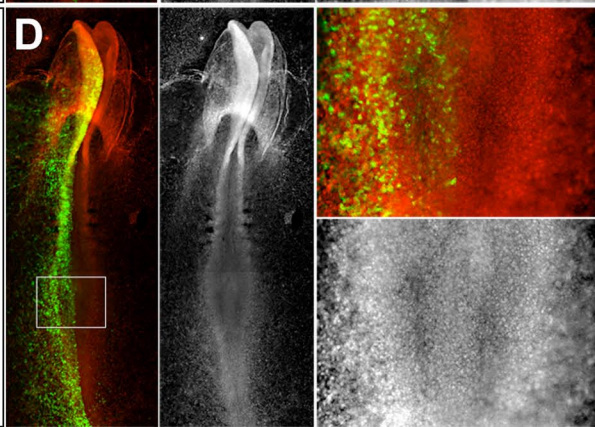


Table S1. Statistical analysis of observed phenotypes

dnFgfr1 pCIG – whole side				
Phenotype	Molecule	Experimental	Control	P value
Expansion	<i>Msx1</i>	8/12	1/12	0.002
	<i>Bmp4</i>	7/8	1/9	<0.001
Inhibition	<i>Gata2</i>	8/11	0/12	<0.001
	<i>Sox2</i>	1/8	0/10	0.351
	<i>Bra</i>	18/20	0/15	<0.001
	<i>Wnt8c</i>	5/6	0/3	0.004
	<i>Tbx6L</i>	10/10	0/9	<0.001
	dpErk	9/9	0/7	<0.001
Pax7 assayed				
Electroporation	Phenotype	Experimental	Control	P value
Stage 3/3+	Inhibited	13/23	0/17	<0.001
	Expanded	6/23	0/17	0.011
Stage 4–	Inhibited	1/11	0/8	0.341
	Expanded	9/11	0/8	0.000
Stage 4	Inhibited	1/10	0/4	0.343
	Expanded	3/10	0/4	0.081
Snail2 assayed				
Electroporation	Phenotype	Experimental	Control	P value
Stage 3/3+	Inhibited	5/9	0/8	0.013
	Expanded	2/9	0/8	0.169
Stage 4–/4	Inhibited	2/8	0/13	0.170
	Expanded	1/8	0/13	0.351
SU5402 beads				
Pax7	Stage grafted	Experimental	Control	P value
Inhibition	Stage 3/3+	7/11	0/3	0.002
	Stage 4–/4	1/10	0/4	0.343
	Stage 4+/-7	0/18	1/12	0.339
Mkp3 pCIG				
Electroporation	Molecule	Experimental	Control	P value
Whole side	<i>Bra</i>	9/9	0/8	<0.001
	<i>Pax7</i>	5/5	0/5	<0.001
pNPB-restricted	<i>Pax7</i>	8/8	0/6	<0.001
pNP-restricted	<i>Pax7</i>	6/7	0/4	<0.001
dnFgfr1 pCIG – pNP restricted				
Phenotype	Molecule	Experimental	Control	P value
Ectopic expression	<i>Pax7</i>	8/8	0/7	<0.001
	<i>Msx1</i>	6/9	0/9	0.004
	<i>Bmp4</i>	4/7	1/9	0.074
Smad6 pCAβ				
Electroporation	Molecule	Experimental	Control	P value
Whole side	pSmad1	4/4	0/3	<0.001
	<i>Gata2</i>	3/3	0/3	<0.001
	<i>Mxs1</i>	3/3	0/3	<0.001
	<i>Pax7</i>	7/7	0/5	<0.001
	<i>Sox2/3</i>	7/7	0/5	<0.001
	<i>Pax7</i>	5/6	0/4	0.004
pNPB-restricted	<i>Pax7</i>	5/6	0/4	0.004
Co-electroporation – pNP restricted Pax7 ectopic expression assayed				
Comparison	Variable 1	Variable 2	P value	
Smad inhibition to control	<u>Smad6+pCIG</u>	<u>pCIG+pCAβ</u>		
	0/4	0/9	>0.999	
FGF inhibition to control	<u>dnFgfr1+pCAβ</u>	<u>pCIG+pCAβ</u>		
	6/8	0/9	0.003	
Smad+FGF inhibition to control	<u>Smad6+dnFgfr1</u>	<u>pCIG+pCAβ</u>		
	2/8	0/9	0.170	
FGF inhibition to Smad+FGF inhibition	<u>dnFgfr1+pCAβ</u>	<u>Smad6+dnFgfr1</u>		
	6/8	2/8	0.049	

Student's *t*-tests of statistical significance, assuming unequal variance were performed, comparing treated and untreated embryos. Headings identify treatment performed. 'Experimental' and 'Control' columns list the total number of embryos displaying the given phenotype. *P* values were calculated comparing experimental and control embryos.