Xenopus Meis3 protein forms a hindbrain-inducing center by activating FGF/MAP kinase and PCP pathways

Emil Aamar and Dale Frank*

Department of Biochemistry, The Rappaport Family Institute for Research in the Medical Sciences, Faculty of Medicine, Technion-Israel Institute of Technology, Haifa 31096, Israel
*Author for correspondence (e-mail: dale@tx.technion.ac.il)

Development 131, 153-163
Published by The Company of Biologists 2004
doi:10.1242/dev.00905

Accepted 6 October 2003

Summary

Knockdown studies in *Xenopus* demonstrated that the *XMeis3* gene is required for proper hindbrain formation. An explant assay was developed to distinguish between autonomous and inductive activities of XMeis3 protein. Animal cap explants caudalized by XMeis3 were recombined with explants neuralized by the BMP dominant-negative receptor protein. *XMeis3*-expressing cells induced convergent extension cell elongations in juxtaposed neuralized explants. Elongated explants expressed hindbrain and primary neuron markers, and anterior neural marker expression was extinguished. Cell elongation was dependent on FGF/MAP-kinase and Wnt-PCP activities. XMeis3 activates FGF/MAP-kinase signaling, which then modulates the PCP pathway. In this manner, XMeis3 protein establishes a hindbrain-inducing center that determines anteroposterior patterning in the brain.

Key words: *Xenopus laevis*, XMeis3, Neural caudalization, Hindbrain-inducing center

Introduction

Meis proteins are involved in cell fate decisions in vertebrate and invertebrate peripheral and central nervous systems (Rieckhof et al., 1997; Kurant et al., 1998; Salzberg et al., 1998; Vlachakis et al., 2001; Maeda et al., 2001; Maeda et al., 2002). Genetic and knockdown studies in *Xenopus* and zebrafish embryos demonstrated a strong requirement for the *Meis3* gene in proper formation and patterning of the hindbrain (Dibner et al., 2001; Waskiewicz et al., 2001; Choe et al., 2002). XMeis3 acted as a potent caudalizer, inducing posterior neural marker expression in both embryos and animal cap explants in the absence of neural induction. Concomitantly, XMeis3 eliminated anterior neural marker expression in embryos and explants (Salzberg et al., 1999). Knockdown of the XMeis3 protein caused a posterior expansion of the forebrain with a simultaneous loss of the hindbrain (Dibner et al., 2001). XMeis3 is expressed in r2-r4 cells, yet hindbrain cell fate is also lost in r5-r7. These results suggest that XMeis3 protein might caudalize the brain in some non-autonomous manner.

Meis proteins form transcriptional complexes by forming dimeric or trimeric DNA-binding complexes with Pbx and or Hox proteins (Shen et al., 1998; Jacobs et al., 1999; Shanmugan et al., 2000; Vlachakis et al., 2000). Through the formation of different complexes, Pbx/Meis protein partners can modify the transcriptional activity of a specific Hox protein in a given cell type. In flies, Meis/HTH is required for the nuclear localization and subsequent DNA binding activity of Pbx/EXD (Rieckhof et al., 1997; Kurant et al., 1998); however, in vertebrates, Meis proteins might not be required for nuclear localization of Pbx proteins (Kilstrup-Nielsen et al., 2003). Work in *Xenopus* and *Drosophila* embryos suggests that Meis proteins act as part of transcriptional activator complexes (Dibner et al., 2001; Inbal et al., 2001). Thus, although Meis proteins may act as nuclear transporters and Hox co-factors, they might actually possess intrinsic transcriptional activator function.

In *Xenopus*, the central nervous system (CNS) is induced in ectoderm by adjacent dorsal mesoderm (Spemann's organizer) during gastrulation. The CNS is characterized by a distinct anteroposterior (A-P) patterning. The predominant model of how A-P patterning is established was suggested by Nieuwkoop (Nieuwkoop, 1952) (reviewed in Doniach et al., 1993). In this two-step model, the initial neural inducing signal specifies anterior neural tissues, such as cement gland and forebrain; this first step is called 'activation'. The second caudalizing step is called 'transformation'. During this step, anterior neural tissue is respecified to more posterior fates, such as midbrain, hindbrain and spinal cord. Several molecules have been identified which participate in the 'activation' and 'transformation' processes. Non-neural ectoderm is induced to anterior-neural tissue by inhibition of bone morphogenetic protein (BMP) activity (reviewed in Harland and Gerhart, 1997). Secreted BMP antagonists bind BMP and inhibit its receptor-binding activity. BMP antagonists are expressed in Spemann's organizer during gastrulation and induce anterior neural tissue in adjacent ectoderm (Harland and Gerhart, 1997). Three secreted factors were shown to caudalize neural tissue in whole embryos or explants: retinoic acid, fibroblast growth factor (FGF) and Xwnt3a (Durston et al., 1989; Sive et al., 1990; Ruiz i Altaba and Jessell, 1991; Sharpe, 1991; Kolm and Sive, 1995; Lamb and Harland, 1995; Cox et al., 1995; McGrew et al., 1995; Blumberg et al., 1997). These pathways appear to interact to caudalize the vertebrate CNS (reviewed in Gamse and Sive, 2000).

A characteristic of posterior neural tissue is its ability to

undergo convergent extension. This typical morphogenesis is restricted to the hindbrain/spinal cord regions and does not occur in the more anterior forebrain/midbrain regions (Keller et al., 1992; Elul et al., 1997). Non-canonical Wnt planar cell polarity (PCP) pathway has been shown to regulate convergent extension in dorsal mesoderm and neuroectoderm of vertebrate and chordate embryos (Wallingford et al., 2000; Heisenberg et al., 2000; Tada and Smith, 2000; Wallingford and Harland, 2001; Keys et al., 2002). Most recently, it has also been shown that FGF signaling might regulate mesodermal and neural cell movements during mouse, chick and frog embryogenesis (Ciruna et al., 2001; Mathis et al., 2001; Yokota et al., 2003). The full nature of the molecular interactions inducing both neural marker expression and morphogenesis in the posterior CNS is still not fully understood.

In this study, we examined how XMeis3 protein mechanistically controls cell fate decisions in the hindbrain. XMeis3 is a transcription factor that could act strictly in a cell-autonomous manner. However, because the *XMeis3* gene is regionally expressed relatively early during development (early to mid-gastrula stages), it is possible that XMeis3 could induce hindbrain by activating non-cell-autonomous caudalizing pathways such as FGF, Wnt or retinoic acid. Our previous studies showed that XMeis3 caudalizing activity is dependent on mitogen-activated-protein kinase (MAPK) activity, presumably through FGF signaling (Ribisi et al., 2000). Two possible explanations could account for this observation. XMeis3 protein could caudalize by direct activation of FGF/MAPK signaling in neuroectoderm cells. Alternatively, basal FGF/MAPK signaling might be required in ectoderm cells as a permissive competence factor that enables caudalization by the XMeis3 protein.

We developed a technique to address the potential inductive non-cell-autonomous role for the XMeis3 protein in neural patterning. In this assay, animal cap ectoderm ectopically expressing XMeis3 protein was recombined with animal cap ectoderm neuralized by ectopic expression of the BMP dominant-negative receptor (DNR) protein. In these experiments, the *XMeis3*-expressing tissue induced mesoderm-independent convergent extension cell elongations in the adjacent juxtaposed neuralized explants. Elongated explants expressed the *Krox20*, *HoxB3* (hindbrain) and *n-tubulin* (primary neuron) markers. Expression of the *otx2* marker (forebrain) was extinguished in the elongated explants. Posterior neural gene expression was dependent on the presence of FGF/MAPK signaling but not canonical or PCP Wnt pathways, whereas convergent extension was dependent on both FGF and PCP activities. In ectoderm explants, ectopic XMeis3 induced *FGF3* expression and subsequent activation of the MAPK pathway. Epistatic experiments suggest that XMeis3 activates FGF/MAPK, which in turn regulates PCP signaling. Thus, XMeis3 protein can act non-cell-autonomously to trigger a signaling cascade, which induces posterior neural marker expression and cell morphogenesis in the developing CNS. *XMeis3*-expressing cells establish a hindbrain induction center that regulates early A-P cell fate in the brain.

Materials and methods

Embryos, explants and in situ hybridization

Ovulation, in vitro fertilization, embryo and explant culture were carried out as described (Re'em-Kalma et al., 1995). Embryos were staged according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1967). Injected and uninjected animal cap explants were removed at the blastula stage using eyebrow knives. We developed a technique to address the potential inductive role for the XMeis3 protein in neural patterning. In this assay, blastula-stage animal cap ectoderm (from a pigmented embryo) ectopically expressing XMeis3 was typically recombined with an albino or lightly pigmented animal cap explant neuralized by ectopic expression of BMP DNR (Fig. 1A). Recombinant explants were aged until sibling embryos reached mid-late neurula stages. Explants were fixed for phenotypic scoring, whole-mount in-situ hybridization and immunostaining (Bonstein et al., 1998). The albino versus pigmented side serves as a lineage trace in the recombinant explants.

RNA, DNA and morpholino-oligonucleotide injections

Various synthetic capped RNAs were injected into the animal hemisphere of embryos at the one-cell stage. Constructs used were: *XMeis3* (Salzberg et al., 1999), *BMP DNR* (Graff et al., 1994), *N17Ras* (Whitman and Melton, 1992), *FGFI DNR* (Amaya et al., 1991), dominant negative *wnt11* (Tada and Smith, 2000), *dsh-xdd1* (Sokol, 1996), *xdsh-D2*, membrane tagged green fluorescent protein (GFP), *dsh-GFP* (Wallingford et al., 2000) and *Gsk-3* (He et al., 1995). Plasmid containing the *3X/(TCF)-luciferase* reporter gene (Nishita et al., 2000) was injected at a concentration of 50 pg per embryo. A mutant non-Wnt-responsive version of the reporter construct was also injected in parallel. The antisense morpholino-oligonucleotide (MO) complementing the 5′ region of the *β-catenin* mRNA (Heasman et al., 2000) was purchased from Gene Tools (Corvallis, Oregon, USA, http://www.gene-tools.com/). One-cell embryos were routinely injected with 10 ng MO in a 5 nl volume. In whole embryos, this MO concentration reproduced the published phenotypes (Heasman et al., 2000).

RT-PCR analysis

Reverse-transcription polymerase chain reaction (RT-PCR) was performed as described previously (Wilson and Melton, 1994), except that random hexamers (100 ng per reaction) were used for reverse transcription. Primers for *EF1α*, *Krox20*, *HoxB9*, *HoxD1* and *XFGF3* were used (Hemmati-Brivanlou and Melton, 1994; Kolm et al., 1997; Domingos et al., 2001).

Western blot, immunostaining and luciferase analysis

MAPK (ERK) western blot analysis was performed using the Phospho-Plus p44/p42 MAPK antibody kit (Cell Signaling / New England Biolabs) as described (Henig et al., 1998). Immunostaining of recombinant animal cap explants and marginal zone explants with Tor70 was performed; tissue culture supernatant of Tor70 antibody was used undiluted (Bolce et al., 1990). Bound antibodies were detected with horseradish-peroxidase (HRP) conjugated secondary antibodies (goat anti-mouse IgG; Pierce) diluted 1:100, and were visualized by HRP staining. After fixation, explants were cleared in 1:2 benzyl alcohol/benzyl benzoate for photography. GFP staining was performed by either fluorescent or immunostaining analysis. Membrane-tagged GFP was detected with a primary anti-GFP antibody (anti-GFP, rabbit IgG, Molecular Probes), followed by a secondary rhodamine-red-conjugated anti-rabbit IgG (H+L) antibody (Jackson Labs). dsh-GFP was analysed by three independent methods. (1) Direct GFP fluorescence. (2) Previously described rhodamine detection. (3) HRP immunohistochemistry. HRP-conjugated secondary goat anti-rabbit (IgG H+L, BioRad) was used. Stained explants were cleared in 1:2 benzyl alcohol/benzyl benzoate for viewing by bright-field or confocal microscopy (Zeiss Axioskop and Radiance 2000, BioRad). Extract preparation and luciferase assays were performed using the Luciferase Assay System (Promega) and activity was measured by integrating total light emission over 30 seconds using a Berthold

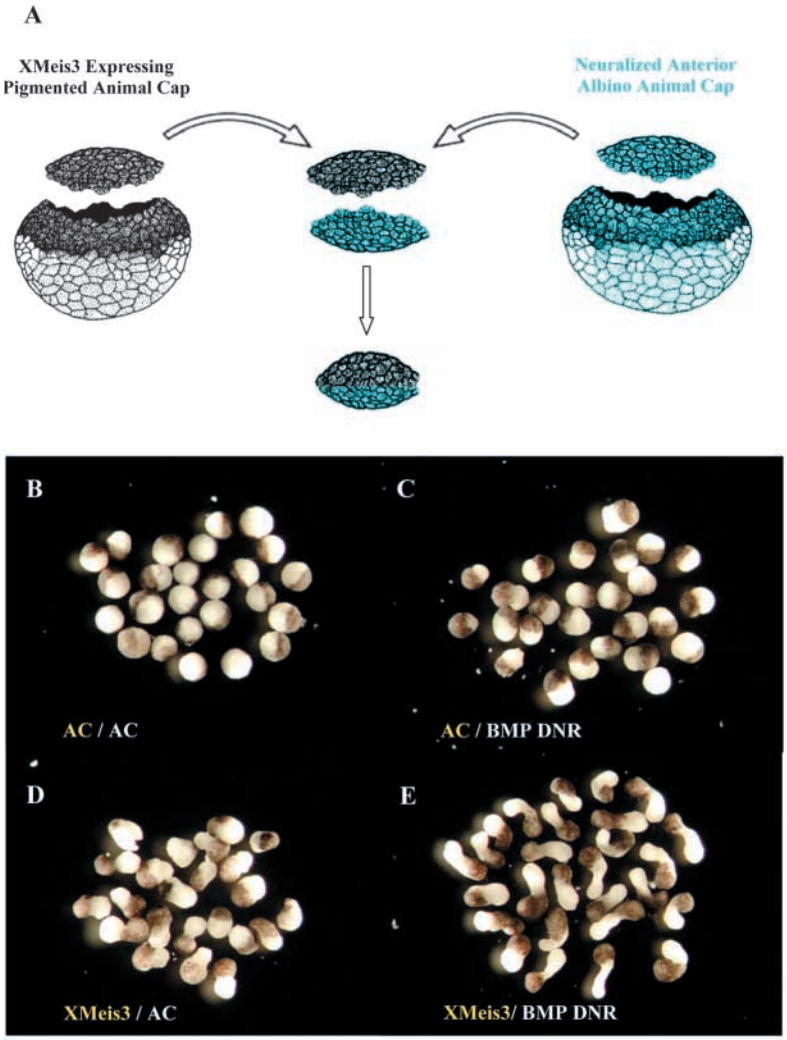


Fig. 1. XMeis3-expressing cells juxtaposed to neuralized tissue induce elongation in a non-autonomous manner. (A) Schematic drawing of recombinant explant strategy. In this system, a pigmented animal cap (AC) explant taken from an embryo ectopically expressing XMeis3-encoding RNA is recombined with an albino AC taken from an embryo ectopically expressing BMP-DNR-encoding RNA. Uninjected pigmented AC explants were recombined with uninjected albino AC explants (B) or with albino explants from embryos injected with BMP-DNR-encoding RNA (0.2-0.4 ng per embryo) (C). Pigmented AC explants expressing XMeis3-encoding RNA (0.8-1.0 ng per embryo) were recombined with uninjected AC explants (D) or with explants expressing BMP-DNR-encoding RNA (E). AC explants were removed from blastula embryos (stage 8-9), recombined and cultured until late neurula (stages 18-19).

luminometer. Luciferase activity was normalized to total protein concentration.

Results

XMeis3 caudalizes in an inductive non-autonomous manner

To determine how XMeis3 controls pattern formation in the *Xenopus* CNS, we developed an assay to distinguish between autonomous and inductive activities of XMeis3 protein. In this system, we typically recombined two heterologous types of animal cap ectoderm tissue. At the one cell stage, pigmented embryos were injected with RNA encoding XMeis3 protein; in parallel, albino embryos were injected with RNA encoding the BMP DNR protein. At blastula stages, these two animal cap explant types were recombined and subsequently cultured to later neurula stages (Fig. 1A).

Explant phenotypes showed that XMeis3 acts inductively. The XMeis3-injected pigmented explant induced cell elongations in the adjacent neuralized albino explant, unlike uninjected control recombinant explants (Fig. 1B,E). Elongation occurred at an average frequency of ~70%, and did not occur (<5%) when uninjected control animal caps were juxtaposed to neuralized animal caps (Fig. 1C) or when XMeis3-expressing animal caps were recombined with control animal caps (Fig. 1D). We have examined over 500 explants in each experimental group.

In addition to elongation analysis, in situ hybridization to hindbrain (*Krox20* r3/r5 and *HoxB3* r5/r6) (Bradley et al., 1992; Godsave et al., 1998) and forebrain (*otx2*) (Blitz and Cho, 1995) markers was also performed in these recombinant explants. In the elongated albino explants, *Krox20* and *HoxB3* expression was induced in comparison to control explants (Fig. 2C-D, Fig. 3A). Expression of *otx2* was high in the anteriorized albino cells in the control recombinant explants (Fig. 2A), but *otx2* expression was extinguished in the elongated explants (Fig. 2B). When *otx2* expression was detected in recombinant explants, it was always expressed in the distal tip of the explant, the furthest distance from the *XMeis3*-expressing cells (Fig. 2B). In the few explants that failed to elongate (Fig. 2B, asterisks), *otx2* expression was normal, thus resembling control recombinant explants, which did not express *XMeis3* (Fig. 2A). In some cases, *Krox20* expression appeared in two stripes, perhaps mimicking the normal r3/r5 pattern (Fig. 2E). *HoxB3* expression always appeared in one stripe, deep within the elongating explant, resembling the endogenous r5/r6 expression pattern (Fig. 3A). Panneural marker (*nrp1*) expression was unaltered in recombinant explants (not shown). Expression of the neurogenic marker *n-tubulin* (Hollemann et al., 1998) was also activated in the elongated explants (Fig. 3B); generally, XMeis3 activity and BMP antagonism alone are weak activators of *n-tubulin* expression in isolated animal cap explants (not shown). These results suggest that neuron cell fate specification was also induced in neuralized cells by *XMeis3*-expressing cells, thus complementing the observation that there is a large reduction of *n-tubulin* expression in XMeis3 knockdown embryos (Dibner et al., 2001).

XMeis3-expressing cells induce cell morphogenesis resembling convergent extension movements of spinal cord/hindbrain cells in neuralized albino explants. To demonstrate that these movements are indeed convergent

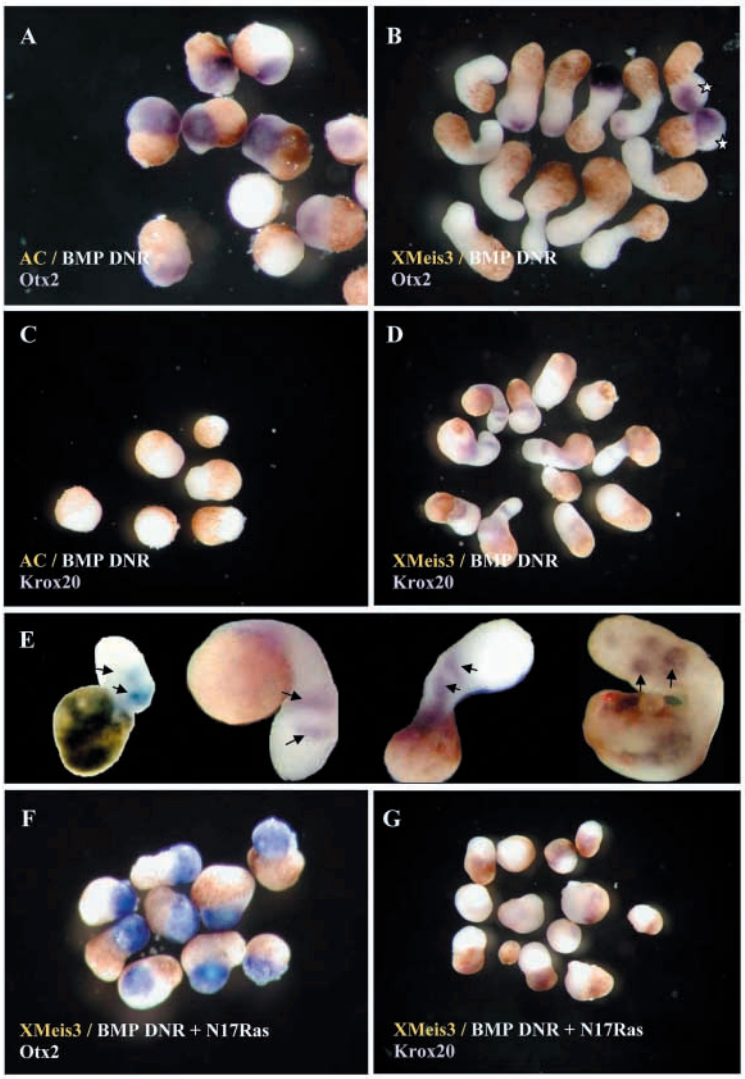


Fig. 2. XMeis3-expressing cells caudalize adjacent anterior neural fated cells. *XMeis3*-injected (1.0 ng RNA) AC explants were taken from pigmented embryos and recombined with AC explants removed from albino embryos neuralized with the *BMP DNR* (0.2 ng RNA). In situ hybridization was performed to the *otx2* (forebrain) and *Krox20* (hindbrain) markers. (A) AC/BMP DNR explants. Expression of *otx2* is high (*n*=7/12) in the neuralized albino side of these recombinant explants. (B) XMeis3/BMP DNR explants. Expression of *otx2* is strongly inhibited (80%, *n*=11/14) in the albino side, and these cells undergo elongation. Notice that residual *otx2* expression is detected at the distal end of the elongated explants and high expression is detected in non-elongated explants (14%, 2/14, asterisks). (C) AC/BMP DNR explants. No elongation or *Krox20* expression is detected (*n*=0/6) on the albino side. (D) XMeis3/BMP DNR explants. *Krox20* expression (67%, *n*=8/12) is induced in the elongating albino side. (E) In some elongating albino explants, two *Krox20* stripes are detected, similar to the endogenous r3/r5 pattern in the embryo. (F,G) XMeis3/BMP DNR explants in which N17Ras- (1.0 ng) and BMP-DNR-encoding RNAs were co-injected on the albino side. In these explants, elongation is inhibited. (F) Expression of *otx2* is rescued (100%; *n*=9/9). (G) *Krox20* expression is inhibited in all of the explants (*n*=12/12).

extension, the BMP-DNR-expressing albino explant was co-injected with an RNA encoding membrane-localized GFP. By immunohistochemistry and confocal microscopy, GFP was used as a tag to delineate cell membrane borders in the elongated explant. As a positive control for convergent extension, elongating neurula-stage dorsal marginal zone (DMZ) explants injected with GFP were also examined. In the DMZs, cells underwent mediolateral intercalation, in contrast to non-elongated animal cap cells (Fig. 4A,B). Like the DMZ explant, many of the cells in the elongated recombinant albino explant underwent mediolateral intercalations in comparison to the control explants (Fig. 4A,C-D). Using this same lineage trace technique, we have observed that GFP/XMeis3-expressing cells in the pigmented animal cap explant do not migrate to the elongating albino side (data not shown), confirming that the elongating neuralized cells are not derived from cells in the *XMeis3*-expressing explant.

We have also ruled out the possibility that the *XMeis3*-expressing pigmented cells are simply inducing de novo *XMeis3* expression in the adjacent albino explant. *XMeis3* mRNA is not detected on the albino side as determined by RT-PCR analysis; in addition, expression of the *XMeis3* MO in the albino explant does not inhibit elongation (data not shown). We also determined if XMeis3 was inducing mesoderm convergent extension by examining mesodermal marker expression in elongating explants. By RT-PCR analysis, we determined that muscle actin was not expressed in recombinant explants (not shown). We also examined expression of the notochord-specific Tor70 marker by immunohistochemistry (Bolce et al., 1992). Although Tor70 was detected in control DMZ explants (Fig. 3C), it was never detected in the recombinant explants (Fig. 3D). The lack of muscle actin or Tor70 expression demonstrated that these elongations are neural in origin. These results support previous data showing that XMeis3 protein caudalizes neural tissue in the absence of mesoderm (Salzberg et al., 1999). Thus, in recombinant explants, the activation of hindbrain and neuron marker expression and the concomitant repression of anterior neural marker expression were also accompanied by hindbrain characteristic convergent extension morphogenesis.

XMeis3 activates a caudalizing MAP kinase activity

XMeis3 caudalizes in a non-cell-autonomous manner, so we wanted to determine whether XMeis3 acts through the FGF/MAPK pathway. Our previous studies showed that XMeis3 protein could not caudalize in the presence of MAPK pathway inhibitors such as dominant-negative Ras (N17Ras) or MAPK phosphatase proteins (Ribisi et al., 2000).

We determined whether XMeis3 protein could activate the FGF/MAPK pathway in animal cap explants. One-cell embryos were injected with RNA encoding the XMeis3 protein and blastula-stage animal cap explants were removed and cultured until early to late gastrula stages. Protein extracts isolated from the animal caps were assayed for phosphorylated ERK (activated MAPK) induction by western analysis (Fig. 5A). XMeis3 transiently induced significant levels of phosphorylated ERK in animal cap explants (a tenfold

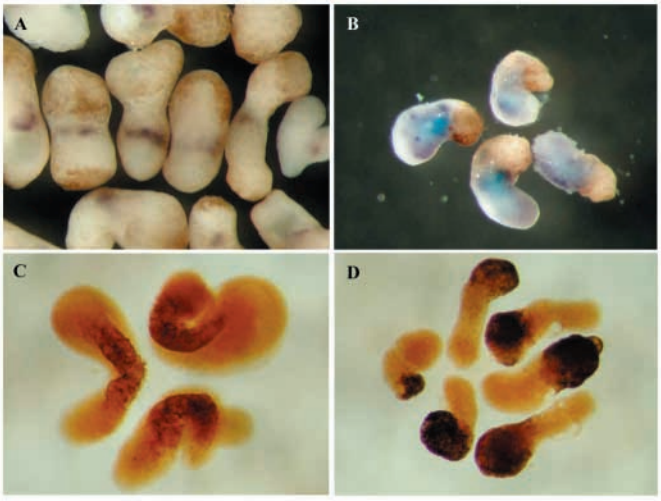


Fig. 3. XMeis3 induces hindbrain and neurogenic, but not mesodermal, marker expression. (A) In situ hybridization to the r5/r6 *HoxB3* gene. *HoxB3* is expressed in >70% of the elongating BMP-DNR-expressing explants (*n*=17/24). XMeis3-expressing AC explants recombined with a control AC explants did not express *HoxB3* (not shown; *n*=0/13). (B) In situ hybridization to the neurogenic *n-tubulin* gene, which is expressed in >50% of the elongating BMP-DNR-expressing explants (*n*=18/34). XMeis3-expressing explants recombined with neither a control AC explant nor an uninjected control AC explant recombined with a BMP-DNR-expressing explant expressed *n-tubulin* (*n*=0/53; not shown). (C) Immunohistochemistry to the notochord-specific Tor70 marker. The elongated DMZ explants stains strongly (*n*=8/8) in comparison to (D) the elongated XMeis3/BMP DNR explants (*n*=0/18). Some non-specific background is seen in the unbleached pigmented animal cap cells.

increase) in comparison to controls (Fig. 5A,B). These levels declined by late neurula stages (not shown). To determine whether ERK activation is dependent on FGF secretion through an active FGFI receptor, one-cell embryos were co-injected with *XMeis3*- and *FGFI-DNR*-encoding RNAs. In these explants, phosphorylated ERK levels were very reduced (Fig. 5A,B) and these explants also failed to express posterior neural markers (Fig. 5C). These results show that XMeis3 could caudalize the CNS by inducing MAPK activation through FGF secretion. Complementing these results, ectopic XMeis3 levels activated *XFGF3* (Fig. 5D) and *XFGF8* (not shown) expression in neurula stage animal cap explants. Interestingly, *XFGF3* (Lombardo et al., 1998) expression overlaps with *XMeis3* in the early neurula *Xenopus* hindbrain r4 region. To verify a role for FGF/MAPK signaling in non-cell-autonomous XMeis3 caudal induction, RNA encoding the N17Ras protein was co-injected with RNA encoding BMP DNR in the recombinant explant assay. In comparison to controls, *XMeis3*-expressing animal cap explants did not induce elongation (Fig. 2F,G) and also did not induce *Krox20* expression (Fig. 2F) in adjacent explants expressing the N17Ras protein. Also, *otx2* expression was not extinguished in these explants (Fig. 2G). Similar to N17Ras, co-injection of FGFI DNR also inhibited elongation and *Krox20* expression in neuralized recombinant explants (not shown). Thus, XMeis3 caudalizes juxtaposed anterior neuroectoderm via the FGF/MAPK signaling pathway.

Role for Wnt signaling in XMeis3 caudalizing induction activity

Canonical and non-canonical Wnt pathways were shown to be involved in posterior neural patterning and morphogenesis in *Xenopus* embryos and explants. Wnt3a and β-catenin were shown to act as potent caudalizers of the *Xenopus* CNS (McGrew et al., 1995; McGrew et al., 1997). Furthermore, canonical Wnt signaling and FGF signaling pathways are thought to interact to posteriorize the *Xenopus* CNS (McGrew et al., 1997; Domingos et al., 2001).

The Wnt-PCP pathway regulates convergent extension in both mesoderm and neural cells (reviewed in Wallingford et al., 2002). We were curious about whether PCP components regulate convergent extension in recombinant explants. Elongation was strongly inhibited in recombinant explants injected with RNAs encoding dominant-negative Wnt 11 protein or PCP dominant-inhibitory disheveled (dsh) proteins such as xdd1 and xdsh-D2 (Fig. 6A-D). Unlike the xdd1 mutant protein, which is dominant-inhibitory to the canonical and PCP pathways, the xdsh-D2 mutant protein still maintains the ability to activate canonical Wnt signaling. Because the xdsh-D2 protein inhibited explant elongation, canonical Wnt activity alone cannot be enough to induce convergent extension in the absence of PCP activity. These results suggest that XMeis3-induced explant elongation is dependent on an active PCP pathway, but independent of canonical Wnt signaling.

This conclusion was further supported by experiments in which canonical β-catenin activity was antagonized by ectopic *GSK-3* expression or by injection of the β-catenin antisense MO (Heasman et al., 2000). Neither ectopic *GSK-3* expression (not shown) nor the β-catenin MO injection inhibited explant elongation (Fig. 7A-C). To address further a role for canonical Wnt signaling in XMeis3-dependent caudalization, the BMP-DNR-expressing explant was co-injected with a β-catenin-sensitive luciferase (luc) reporter gene construct *3X(TCF)/Luc* (Nishita et al., 2000). In the positive control for β-catenin driven luc activity, the *XMeis3*-expressing explant was fused to an uninjected naive (non-elongating) animal cap explant expressing the reporter construct (Fig. 7D, left bar). In parallel with this, *XMeis3*-expressing explants were fused to *BMP DNR* neuralized explants (plus reporter construct), +/– ectopic *GSK-3* expression. In the elongating explants expressing only BMP DNR protein, a 40% reduction of β-catenin reporter activity was observed (Fig. 7D, middle bar). In the presence of GSK-3, luc activity was reduced by over 90% (Fig. 7D, right bar) and these explants elongated normally (not shown, similar to Fig. 7C). Experiments in isolated animal cap explants complemented these observations. Animal caps co-injected with XMeis3- and GSK-3-encoding RNAs expressed *Krox20*- and *HoxB9*-like explants expressing *XMeis3* alone (Fig. 7E); however, 95-98% of the β-catenin-dependent luc activity was inhibited in these *XMeis3/GSK-3*-injected explants (not shown). Thus, large reductions in luc activity were not associated with any inhibitory effect on elongation in the recombinant explant assay or posterior neural marker expression in isolated animal cap explants. Thus, XMeis3 caudalizing activity is not highly dependent on the canonical β-catenin pathway, in sharp contrast to FGF/MAPK signaling. Similarly, isolated animal cap explants co-expressing RNAs encoding the dominant-negative *Wnt11* ligand and *XMeis3* did

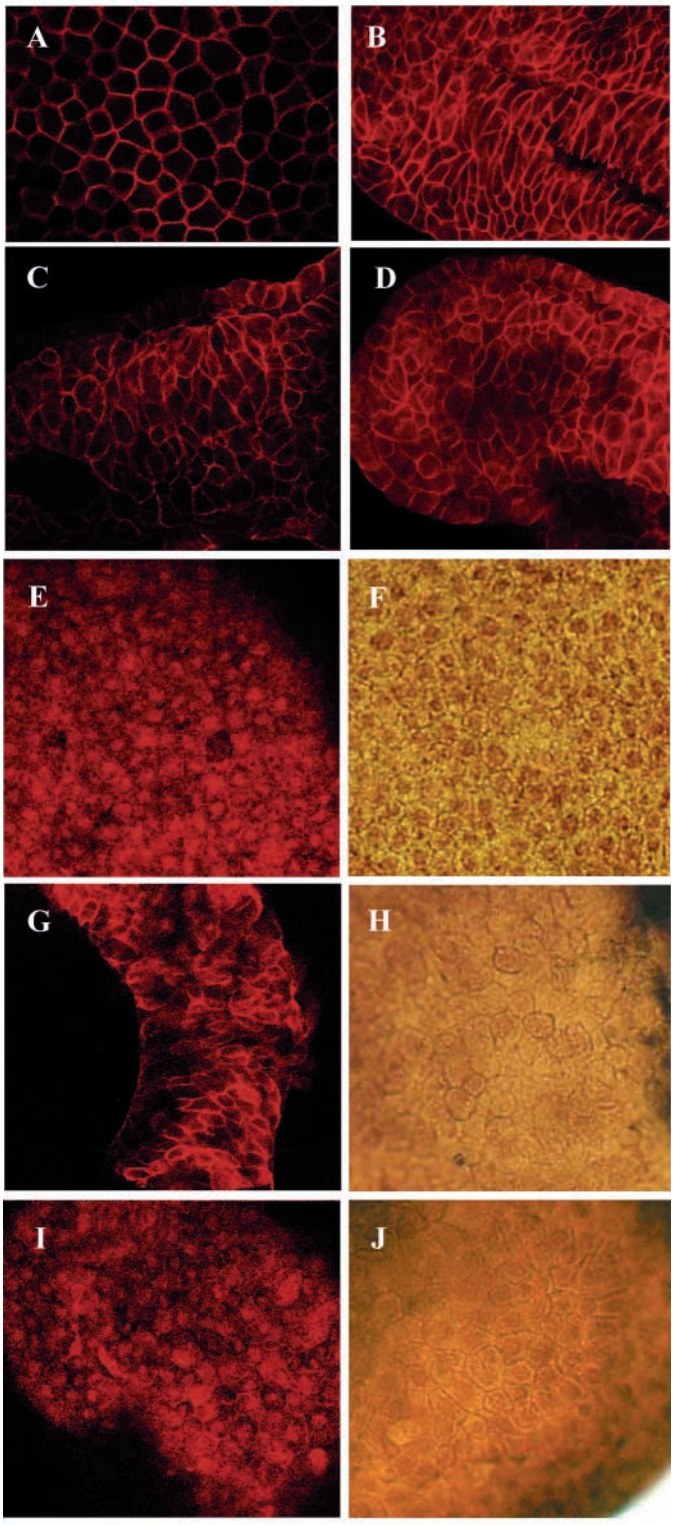


Fig. 4. XMeis3 induces convergent extension and membrane localization of dsh protein in recombinant explants. (A) AC/AC recombinant explant in which the albino side is injected with RNA (50 pg) encoding membrane-localized GFP. (B) DLMZ albino explant injected with membrane-localized GFP. The explant has undergone convergent extension and cells are intercalated. (C,D) XMeis3/BMP DNR recombinant explants in which the elongated BMP DNR albino side is co-injected with membrane-localized GFP. Cells in the elongated side have intercalated and undergone convergent extension in comparison to control explants (A). (E) AC/BMP DNR recombinant explant (late neurula) injected with RNA encoding dsh-GFP (50 pg) on the albino side (rhodamine detection). The dsh-GFP is detected diffusely throughout the cells in the non-elongating explant. (F) AC/BMP DNR recombinant explant (early neurula, pre-elongation) injected with dsh-GFP on the albino side (HRP detection). The dsh-GFP is detected diffusely throughout the cells in the explant. (G) XMeis3/BMP DNR recombinant explant (late neurula) injected with dsh-GFP on the albino side (rhodamine detection). The dsh-GFP is localized to the intercalated elongating cells. (H) XMeis3/BMP DNR recombinant explant (early neurula, pre-elongation) injected with dsh-GFP on the albino side (HRP detection). The dsh-GFP is membrane localized. (I) XMeis3/BMP + FGFI DNR recombinant explant (late neurula) injected with dsh-GFP on the albino side (rhodamine detection). The dsh-GFP is detected diffusely throughout the cells in the non-elongating explant. (J) XMeis3/BMP + FGFI DNR recombinant explant (early neurula, pre-elongation) injected with dsh-GFP on the albino side (HRP detection). The dsh-GFP is detected diffusely throughout the cells in the explant.

not have reduced expression of posterior neural markers such as *Krox20* and *HoxD1* (Fig. 6E). Both PCP and FGF/MAPK pathway inhibition prevented explant elongation but, in contrast to MAPK signaling inhibition, PCP antagonism did not perturb induction of posterior neural marker expression by XMeis3.

PCP pathway is downstream of FGF/MAPK signaling

Elongation in recombinant explants was dependent on both FGF/MAPK and PCP pathways. We therefore investigated the hierarchical relationship of these two pathways. The BMP DNR neuralized recombinant explants were co-injected with RNA encoding the FGFI DNR protein and a dsh-GFP fusion protein. Previous studies have shown that, when expressed at low levels, dsh-GFP mimics endogenous dsh and undergoes membrane localization in the presence of PCP activity (Wallingford et al., 2000). In elongated explants, we found that dsh-GFP did indeed undergo membrane localization in comparison to control explants (Fig. 4E-H); these same cells have undergone medial intercalations characteristic of convergent extension (Fig. 4E,G). In non-elongating explants expressing the BMP DNR and FGFI DNR proteins, dsh-GFP was not membrane localized (Fig. 4I,J), thus resembling the non-elongating control explants (Fig. 4E,F). This result strongly suggests that PCP activity (dsh membrane localization) requires upstream FGF signaling.

These results were supported by additional experiments in which BMP DNR neuralized recombinant explants were co-injected with FGFI DNR protein and the PCP constitutive activating form of Daam-1 protein (C-Daam-1). Daam-1 links dsh to the membrane and its inhibition blocked convergent extension in embryos and activin-treated animal cap explants (Habas et al., 2001). C-Daam-1 rescued the antagonistic effects of dominant inhibitory PCP proteins on activin-mediated elongations in animal cap explants (Habas et al., 2001). We found that ectopic expression of *C-Daam-1*-encoding RNA partially rescued cell elongations in neuralized explants that were inhibited by the FGFI DNR protein (not shown).

These results place the PCP pathway downstream of FGF

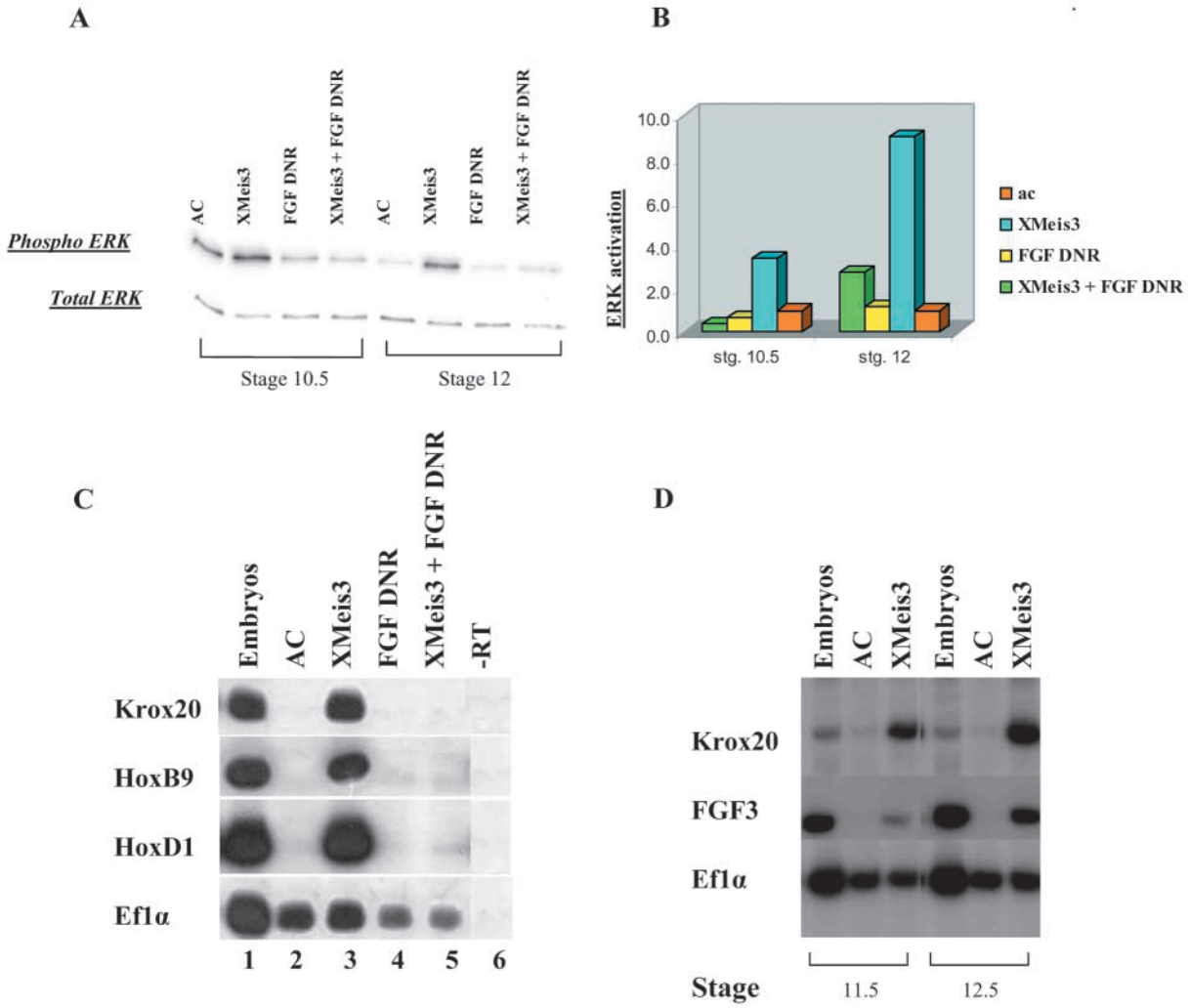


Fig. 5. XMeis3 activates FGF/MAPK caudalizing activities. (A) Embryos at the one-cell stage were injected with 1.0 ng of XMeis3-encoding RNA, 1.0 ng of FGFI-DNR-encoding RNA or both. Animal cap explants removed at blastula stages were grown to early and late gastrula stages. Protein was isolated and western blot analysis of phosphorylated-ERK and total ERK protein was performed. One representative experiment is shown. (B) The bar graphs for (A) describe chemiluminescent quantitation of samples performed using an Image Maker VPS-CL monitor (Amersham-Pharmacia). To quantify the samples, the relative amounts of phosphorylated ERK and total ERK were calculated and plotted on the bar graphs. (C) One-cell-stage embryos were injected in the animal hemisphere with 1.0 ng of *XMeis3* RNA, 1.0 ng of *FGF1 DNR* RNA or both. 18 animal cap explants were removed from uninjected and injected groups of blastula embryos (stage 8-9). Explants from each group were grown to stage 20 and total RNA was isolated. RT-PCR analysis was performed with the markers *Krox20*, *HoxB9* and *HoxD1*. *EF1α* served as a control for quantifying RNA levels in the different samples. For controls, RT-PCR and –RT-PCR was performed on total RNA isolated from normal embryos. (D) One-cell-stage embryos were injected in the animal hemisphere with 1.0 ng *XMeis3* RNA. 36 animal cap explants were removed from uninjected and injected groups of blastula embryos (stage 8-9). 18 explants from each group were grown to stages 11.5 and 12.5, and total RNA was isolated. RT-PCR analysis was performed with the markers *Krox20* and *FGF3*. *EF1α* served as a control to quantify RNA levels in the different samples. For controls, RT-PCR and –RT-PCR (not shown) were performed on total RNA isolated from normal embryos.

signaling. XMeis3 non-cell-autonomous caudalization activity is initiated through FGF signaling, which activates components of the PCP pathway. This induction drives neural convergent extension cell movements.

Discussion

These studies show that XMeis3 caudalizes the CNS in an inductive non-cell-autonomous manner. XMeis3 expressed in pigmented animal cap explants induced cell fate changes in juxtaposed neuralized albino animal cap explants. The XMeis3-expressing explant induced posterior neural and neurogenic marker expression while concurrently inhibiting anterior neural marker expression in the adjacent neuralized explant. Mesodermal markers were not expressed in these recombinant explants. Ectopic XMeis3 expression in pigmented explants induced convergent extension cell movements characteristic of hindbrain cells in adjacent albino cells. Previous studies in isolated animal caps have shown that XMeis3 can activate posterior neural marker expression while simultaneously inhibiting anterior neural marker expression (Salzberg et al., 1999). However, in these isolated explants,

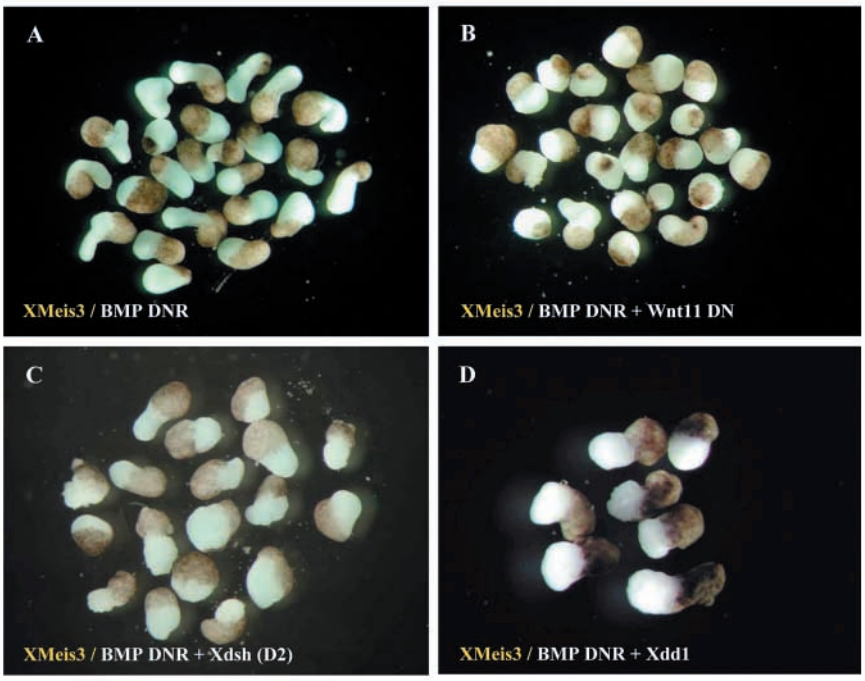
cells did not elongate or undergo differentiation to neuralized hindbrain cells.

In elongated explants, expression of the *Krox20* and *HoxB3* hindbrain markers was induced. These hindbrain markers were expressed deeply within the elongated neural tissue. There was also a concomitant decline in forebrain-specific *otx2* expression. In some elongating explants, *Krox20* was expressed in two stripes, resembling the r3/r5 expression pattern in whole embryos. *HoxB3* was always expressed as a single stripe within the elongating explants, resembling its normal expression pattern in r5/r6. In parallel, *otx2* expression was extinguished in elongated explants or pushed into the most distal tip of the explant, the furthest from the XMeis3-expressing cells. This pattern of neural marker expression in the recombinant explants suggests that XMeis3 induces a rearrangement of neural pattern, which produces an A-P axis in the brain. In the recombinant explants, XMeis3-expressing cells send a signal to adjacent neuralized cells, which appear to recapitulate the normal process of A-P pattern formation ('activation and transformation') in the developing brain. These XMeis3-expressing cells form a hindbrain-inducing center, which can pattern the brain with a well-defined A-P symmetry.

Additionally, *n-tubulin* expression was also activated in the elongated explants. *N-tubulin* is a marker of cells fated to differentiate as neurons. Interestingly, XMeis3 and BMP antagonist activities alone were weak activators of *n-tubulin* expression in animal cap explants, yet in XMeis3 knock down embryos, there was large reduction of *n-tubulin* expression (Dibner et al., 2001). These results suggest that XMeis3-inducing signals interact with neuralized tissue to promote neurogenesis in the developing embryo.

Previous studies in *Xenopus* showed that posterior neural patterning was dependent on an active FGF/MAPK signaling (Holowacz and Sokol, 1999; Ribisi et al., 2000). Furthermore, we also showed that XMeis3 could not activate the expression of posterior neural markers in isolated animal cap explants co-injected with MAPK pathway inhibitors (Ribisi et al., 2000). XMeis3 appears to activate the FGF/MAPK pathway as part of its caudalizing program. XMeis3 activated *XFGF3* and *XFGF8* (not shown) expression in isolated early-neurula-stage animal cap explants. In these same explants, *XMeis3* expression induced high phosphorylated-ERK levels, in comparison to control explants. XMeis3 activation of ERK was dependent on the presence of an active FGFI receptor. In recombinant explants, FGF signaling antagonist molecules expressed in the neuralized explant side inhibited convergent extension and *Krox20* expression mediated by juxtaposed *XMeis3*-expressing cells; in these same recombinant explants *otx2* expression was not inhibited. These results demonstrate that XMeis3 non-cell-autonomously caudalizes anterior neuroectoderm via the FGF/MAPK signaling pathway.

XFGF3 is expressed in overlapping r4 regions with *XMeis3* in the early neurula *Xenopus* hindbrain (Lombardo et al., 1998). *FGF3* and *FGF8* are also expressed in the early chick and zebrafish hindbrain (Mahmood et al., 1995; Maves et al., 2002), yet little is known about the expression pattern or role of FGF8 protein in *Xenopus* hindbrain formation. *FGF3/8* expression in r4 was sufficient to organize a hindbrain-inducing center in zebrafish (Maves et al., 2002; Walshe et al., 2002). Our results suggest that XMeis3 organizes this hindbrain-inducing center by activating *FGF3* gene expression and subsequent protein secretion. *XMeis3* is initially expressed



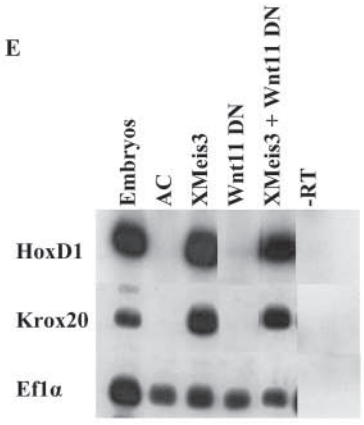


Fig. 6. Inhibition of Wnt PCP activity inhibits XMeis3-induced convergent extension but not neural marker expression. (A) XMeis3/BMP DNR recombinant explants in which the neuralized albino cells undergo elongation (60%, *n*=14/24). (B) XMeis3/BMP DNR explants co-injected in the albino explant with 0.5 ng of *wnt11* dominant-negative ligand (14% weak elongation, *n*=2/24), (C) *Xdsh-(d2)* (22% weak elongation, *n*=4/18) or (D) *Xdd1* (no elongations, *n*=0/18) encoding RNAs. Elongation is inhibited in the neuralized albino side in all of the co-injected groups (B-D). (E) One-cell-stage embryos were injected in the animal hemisphere with 1.0 ng of *XMeis3* RNA, 0.5 ng of *wnt11* dominant-negative ligand RNA or both. 18 animal cap explants were removed from uninjected and injected groups of blastula embryos (stage 8-9). Explants from each group were grown to stage 20 and total RNA was isolated. RT-PCR analysis was performed with the markers *Krox20* and *HoxD1*. *EF1α* served as a control to quantify RNA levels in the different samples. For controls, RT-PCR and –RT-PCR were performed on total RNA isolated from normal embryos.

in a stripe of cells in the presumptive hindbrain of late gastrula embryos, in the proper time and place to activate this induction center. The observation that XMeis3 acts via FGF in a non-cell-autonomous manner probably explains why hindbrain cells outside the r2-r4 *XMeis3* expression domain are lost in embryos expressing the XMeis3 MO or antimorph protein (Dibner et al., 2001). The loss of XMeis3 activity in r2-4 eliminates this induction center and so caudalization of the anterior CNS and pattern formation of the whole hindbrain are disrupted.

Embryos knocked down by the XMeis3 antimorph protein or MO have a significantly shortened body axis, strongly suggesting that neural convergent extension in the hindbrain has been inhibited (Dibner et al., 2001). XMeis3 knockdown embryos have very similar phenotypes to embryos in which the Wnt-PCP pathway was inhibited in the neural plate (Wallingford and Harland, 2001). Supporting this conclusion, we have observed that neural plate explants (cultured from late gastrula to late neurula stages) expressing the *XMeis3* MO elongate significantly less than normal explants (E.A. and D.F., unpublished).

Canonical and non-canonical Wnt pathways have been shown to be involved in posterior neural patterning and morphogenesis in vertebrate and chordate embryos and explants. The canonical Wnt pathway was shown to caudalize the *Xenopus*, chick and zebrafish CNS (McGrew et al., 1995; Kiecker and Niehrs 2001; Domingos et al., 2001; Nordstrom et al., 2002; Kudoh et al., 2002). Canonical Wnt and FGF signaling pathways apparently interact to pattern the vertebrate CNS (McGrew et al., 1997; Domingos et al., 2001; Kudoh et al., 2002; Nordstrom et al., 2002), whereas Wnt-PCP activities regulate convergent extension cell movements in both mesoderm and posterior neural cells (Wallingford et al., 2002).

We determined whether PCP or canonical Wnt components were involved in the cell elongations detected in the recombinant explants. In recombinant explants injected with RNAs encoding PCP dominant-inhibitory proteins, convergent extension was inhibited. Elongations induced by XMeis3 in the adjacent neuralized explants were dependent on the PCP pathway but not the canonical-Wnt pathway, and β-catenin activity could not trigger cell elongations in the absence of the PCP pathway. Antagonism of β-catenin activity did not inhibit elongation in the neuralized explant or the activation of posterior neural markers by XMeis3 in isolated animal cap explants. This result suggests that XMeis3 induces posterior neural marker expression independently of canonical Wnt signaling. XMeis3 induces PCP activation in neuralized cells, because dsh-GFP protein was localized to the membranes of elongating recombinant explants only in the presence of adjacent XMeis3-expressing cells. In recombinant explants lacking XMeis3, dsh-GFP was found in a diffuse cytoplasmic manner. In isolated injected animal cap explants, inhibition of PCP activity had no drastic effect on posterior neural marker expression induced by XMeis3. Unlike FGF/MAPK pathway inhibition, in which XMeis3 induction of both posterior marker expression and elongation were strongly inhibited, PCP activity modulation prevented explant elongation but did not significantly alter posterior neural marker expression by XMeis3.

Convergent extension in the recombinant explants was

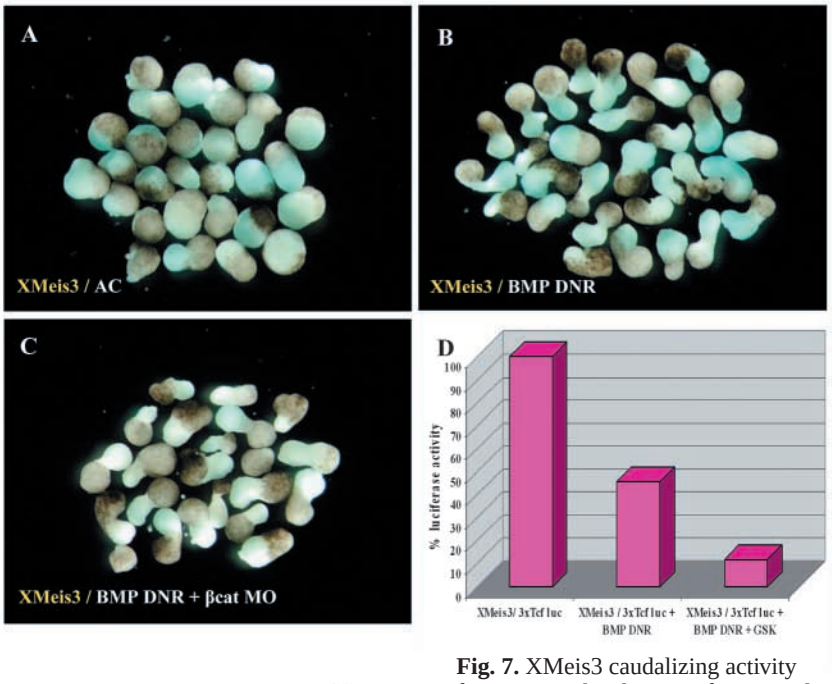


Fig. 7. XMeis3 caudalizing activity functions in the absence of canonical Wnt signaling. All embryos were injected in the animal hemisphere with RNA or DNA at the one-cell stage and animal caps were removed at blastula stages 8-9. (A) XMeis3/AC control recombinant explants (no elongations, *n*=0/28). (B) XMeis3/BMP DNR recombinant explants, in which the neuralized albino cells undergo elongation (83% elongations, *n*=25/30). (C) XMeis3/BMP DNR explants co-injected in the albino neuralized explant with *β-catenin* MO. These explants elongate (85% elongation, *n*=17/20). (D) Albino explants were also co-injected with the β-catenin-promoter-driven luciferase reporter construct *3X TCF-luc* on the albino side as described in (A,B), except that 0.5 ng GSK-3-encoding RNA was injected instead of *β-catenin* MO. At early neurula stages, 8-15 explants were lysed per group and luciferase activity assayed. The graph shows relative luciferase activity in each sample, with the control (A) taken as 100% luciferase activity. (E) One-cell-stage embryos were injected in the animal hemisphere with 1.0 ng *XMeis3* RNA, 0.5 ng *GSK-3* RNA or both. 18 animal cap explants were removed from uninjected and injected groups of blastula embryos (stage 8-9). Explants from each group were grown to stage 20 and total RNA was isolated. RT-PCR analysis was performed with the markers *Krox20* and *HoxB9*. *EF1α* served as a control to quantify RNA levels in the different samples. For controls, RT-PCR and –RT-PCR was performed on total RNA isolated from normal embryos.

dependent on both FGF/MAPK and PCP pathways. The epistatic relationship of these two pathways was determined. The BMP DNR/dsh-GFP neuralized recombinant explants were co-injected with FGFI-DNR-encoding RNA. In the absence of FGF antagonism, dsh was membrane localized in the elongated explants; in the presence of FGFI DNR, explants did not elongate and dsh was not detected in the membrane. This observation suggests that FGF/MAPK signaling lies upstream of the PCP pathway and that dsh localization to the membrane is dependent upon functional FGF signaling. Further supporting these results, we showed that FGFI DNR inhibition of XMeis3-induced cell elongations could be partially rescued in explants expressing the PCP constitutive activated form of the Daam-1 protein. Daam-1 is a PCP component that links dsh to the membrane (Habas et al., 2001).

A role for FGF signaling in early vertebrate mesodermal and neural morphogenesis has been suggested (Ciruna et al., 2001; Mathis et al., 2001), and most recent experiments in dorsal mesoderm explants suggest that convergent extension cell movements are regulated by FGF signaling (Yokota et al., 2003). Our results place the PCP pathway downstream of FGF signaling in regulating neural convergent extension. XMeis3 non-cell-autonomous caudalization activity is initiated through FGF signaling, which activates the PCP pathway, leading to membrane localization of the dsh protein in the neuralized explant. Thus, an XMeis3 induction center in r2-r4 organizes hindbrain formation; this center induces activation of FGF-dependent posterior neural marker expression and morphogenetic cell movements characteristic of the hindbrain. Further studies will determine which caudalizing components are required for the expression of neural markers along the A-P axis in comparison to the components regulating neural cell movements. Investigation is needed to elucidate how XMeis3 coordinates FGF signaling, the three Wnt pathways (canonical, PCP and calcium) and retinoid activities in order to make a hindbrain.

We thank R. Habas, X. He, J. Wallingford, J. Smith and R. Harland for plasmids, J. Yisraeli for the GFP immunostaining protocol, and O. Shenkar and A. Salzberg for valuable microscopy assistance. This work was supported by grants from the Israel Cancer Research Fund, the Israel Science Foundation founded by the Israel Academy of Sciences and Humanities and the Technion Fund for the Advancement of Research. E.A. was generously supported by a ‘Kol Kore’ predoctoral scholarship from the Israel Ministry of Science, Culture and Sport.

References

Amaya E., Musci, T. J. and Kirschner, M. W. (1991). Expression of a dominant negative mutant of the FGF receptor disrupts mesoderm formation in *Xenopus* embryos. *Cell* **66**, 257-270.

Blitz, I. L. and Cho, K. W. (1995). Anterior neuroectoderm is progressively induced during gastrulation: the role of the *Xenopus* homeobox gene *orthodenticle*. *Development* **121**, 993-1004.

Blumberg, B., Bolado, J., Moreno, T., Kintner, C., Evans, R. M. and Papalopulu, N. (1997). An essential role for retinoid signaling in anteroposterior neural patterning. *Development* **124**, 373-379.

Bolce, M. E., Hemmati-Brivanlou, A., Kushner, P. D. and Harland, R. M. (1992). Ventral ectoderm of *Xenopus* forms neural tissue, including hindbrain, in response to activin. *Development* **115**, 681-688.

Bonstein, L., Elias, S. and Frank, D. (1998). Paraxial-fated mesoderm is required for neural crest induction in *Xenopus* embryos. *Dev. Biol.* **193**, 156-168.

Bradley, L., Snape, A., Bhatt, S. and Wilkinson, D. (1992) The structure and expression of the *Xenopus Krox-20* gene: conserved and divergent patterns of expression in rhombomeres and neural crest. *Mech. Dev.* **40**, 73-84.

Choe, S. K., Vlachakis, N. and Sagerstrom, C. G. (2002). Meis family proteins are required for hindbrain development in the zebrafish. *Development* **129**, 585-595.

Ciruna, B. and Rossant, J. (2001). FGF signaling regulates mesoderm cell fate specification and morphogenetic movement at the primitive streak. *Dev. Cell* **1**, 37-49.

Cox, W. G. and Hemmati-Brivanlou, A. (1995). Caudalization of neural fate by tissue recombination and bFGF. *Development* **121**, 4349-4358.

Dibner, C., Elias, S. and Frank, D. (2001). XMeis3 protein activity is required for proper hindbrain patterning in *Xenopus laevis* embryos. *Development* **128**, 3415-3426.

Domingos, P. M., Itasaki, N., Jones, C. M., Mercurio, S., Sargent, M. G., Smith, J. C. and Krumlauf, R. (2001). The Wnt/β-catenin pathway posteriorizes neural tissue in *Xenopus* by an indirect mechanism requiring FGF signalling. *Dev. Biol.* **239**, 148-160.

Doniach, T. (1993). Planar and vertical induction of anteroposterior patterning during the development of the Amphibia central nervous system. *J. Neurobiol.* **24**, 1256-1275.

Durston, A., Timmermans, J., Jage, W., Hendeiks, H., deVries, N., Heidveld, M. and Nieuwkoop, P. (1989) Retinoic acid causes an anteroposterior transformation in the developing central nervous system. *Nature* **340**, 140-144.

Elul, T., Koehl, M. A. and Keller R. (1997). Cellular mechanism underlying neural convergent extension in *Xenopus laevis* embryos. *Dev. Biol.* **191**, 243-258.

Gamse J. and Sive H. (2000). Vertebrate anteroposterior patterning, the *Xenopus* neurectoderm as a paradigm. *BioEssays* **11**, 976-986.

Godsave, S. F., Koster, C. H., Getahun, A., Mathu, M., Hooiveld, M., Van Der Wees, J., Hendriks, J. and Durston, A. J. (1998). Graded retinoid responses in the developing hindbrain. *Dev. Dyn.* **213**, 39-49.

Graff, J. M., Thies, R. S., Song, J. J., Celeste, A. J. and Melton, D. A. (1994). Studies with a *Xenopus* BMP receptor suggest that ventral mesoderm-inducing signals override dorsal signals in vivo. *Cell* **79**, 169-179.

Habas, R., Kato, Y. and He, X. (2001). Wnt/Frizzled activation of Rho regulates vertebrate gastrulation and requires a novel Formin homology protein Daam1. *Cell* **107**, 843-854.

Harland, R. and Gerhart, J. (1997). Formation and function of Spemann’s organizer. *Annu. Rev. Cell. Dev. Biol.* **13**, 611-667.

He, X., Saint-Jeannet, J. P., Woodgett, J. R., Varmus, H. E. and Dawid, I. B. (1995). Glycogen synthase kinase-3 and dorsoventral patterning in *Xenopus* embryos. *Nature* **374**, 617-622.

Heasman, J., Kofron, M. and Wylie, C. (2000). β-Catenin signaling activity dissected in the early *Xenopus* embryo; a novel antisense approach. *Dev. Biol.* **222**, 124-134.

Heisenberg, C. P., Tada, M., Rauch, G. J., Saude, L., Concha, M. L., Geisler, R., Stemple, D. L., Smith J. C. and Wilson S. W. (2000). Silberblick/Wnt11 mediates convergent extension movements during zebrafish gastrulation. *Nature* **405**, 76-81.

Hemmati-Brivanlou, A. and Melton, D. A. (1994). Inhibition of activin receptor signaling promotes neuralization in *Xenopus*. *Cell* **77**, 273-281.

Henig, C., Elias, S. and Frank, D. (1998). A POU protein regulates mesodermal competence to FGF in *Xenopus*. *Mech. Dev.* **71**, 131-142.

Hollemann, T., Chen, Y., Grunz, H. and Pieler, T. (1998). Regionalized metabolic activity establishes boundaries of retinoic acid signalling. *EMBO J.* **17**, 7361-7372.

Holowacz, T. and Sokol, S. (1999). FGF is required for posterior neural patterning but not for neural induction. *Dev. Biol.* **205**, 296-308.

Inbal, A., Halachmi, N., Dibner, C., Frank, D. and Salzberg, A. (2001). Genetic evidence for the transcriptional-activating function of Homothorax during adult fly development. *Development* **128**, 3405-3413.

Jacobs, Y., Schnabel, C. A. and Cleary, M. L. (1999). Trimeric association of Hox and TALE homeodomain proteins mediates Hoxb2 hindbrain enhancer activity. *Mol. Cell. Biol.* **19**, 5134-5142.

Keller, R., Shih, J. and Sater, A. (1992). The cellular basis of the convergence and extension of the *Xenopus* neural plate. *Dev. Dyn.* **193**, 199-217.

Keys, D. N., Levine, M., Harland, R. M. and Wallingford, J. B. (2002). Control of intercalation is cell-autonomous in the notochord of *Ciona intestinalis*. *Dev. Biol.* **246**, 329-340.

Kiecker, C. and Niehrs, C. (2001). A morphogen of Wnt/β-catenin signaling regulates anteroposterior neural patterning in *Xenopus*. *Development* **128**, 4189-4201.

Kilstrup-Nielsen, C., Alessio, M. and Zappavigna, V. (2003). PBX1 nuclear export is regulated independently of PBX-MEINOX interaction by PKA phosphorylation of the PBC-B domain. *EMBO J.* **22**, 89-99.

Kolm, P. J. and Sive, H. (1995). Hindbrain patterning requires retinoid signaling. *Dev. Biol.* **192**, 1-16.

Kolm, P. J., Apekin, V. and Sive, H. (1997). Regulation of *Xenopus* labial homeodomain genes, *HoxA1* and *HoxD1*: activation by retinoids and peptide growth factors. *Dev. Biol.* **167**, 34-49.

Kudoh, T., Wilson, S. W. and Dawid, I. B. (2002). Distinct roles for FGF, Wnt and retinoic acid in posteriorizing the neural ectoderm. *Development* **129**, 4335-4346.

Kurant, E., Pai, C. Y., Sharf, R., Halachmi, N., Sun, Y. H. and Salzberg, A. (1998). Dorsotonals/Homothorax, the *Drosophila* homologue of Meis-1, interacts with Extradenticle in patterning of the embryonic PNS. *Development* **125**, 1037-1048.

Lamb, T. M. and Harland, R. M. (1995). Fibroblast growth factor is a direct neural inducer, which combined with noggin generates anterior-posterior neural pattern. *Development* **121**, 3627-3636.

Lombardo, A., Isaacs, H. V. and Slack, J. M. (1998). Expression and functions of FGF-3 in *Xenopus* development. *Int. J. Dev. Biol.* **42**, 1101-1107.

Maeda, R., Mood, K., Jones, T. L., Aruga, J., Buchberg, A. M. and Daar, I. O. (2001). *Xmeis1*, a protooncogene involved in hindbrain and neural gene expression in *Xenopus* embryos. *Oncogene* **20**, 1329-1342.

Maeda, R., Ishimura, A., Mood, K., Park, E. K., Buchberg, A. M. and Daar, I. O. (2002). Xpbx1b and Xmeis1b play a collaborative involved in specifying neural crest cell fate in *Xenopus* embryos. *Proc. Natl. Acad. Sci. USA* **99**, 5448-5453.

Mahmood, R., Kiefer, P., Guthrie, S., Dickson, C. and Mason, I. (1995). Multiple roles for FGF-3 during cranial neural development in the chicken. *Development* **121**, 1399-1410

Mathis, L., Kulesa, P. M. and Fraser, S. E. (2001). FGF receptor signalling is required to maintain neural progenitors during Hensen's node progression. *Nat. Cell Biol.* **3**, 559-566.

Maves, L., Jackman, W. and Kimmel, C. B. (2002). FGF3 and FGF8 mediate a rhombomere 4 signaling activity in the zebrafish hindbrain. *Development* **129**, 3825-3837.

McGrew, L. L., Lai, C. J. and Moon, R. T. (1995). Specification of the anteroposterior neural axis through synergistic interaction of the Wnt signaling cascade with noggin and follistatin. *Dev. Biol.* **172**, 337-342.

McGrew, L. L., Hoppler, S. and Moon, R. T. (1997). Wnt and FGF pathways cooperatively pattern anteroposterior neural ectoderm in *Xenopus*. *Mech. Dev.* **69**, 105-114.

Nieuwkoop, P. (1952) Activation and organization of the central nervous system in amphibians III. Synthesis of a new working hypothesis. *J. Exp. Zool.* **120**, 83-108.

Nieuwkoop, P. and Faber, J. (1967). *Normal Table of* Xenopus laevis *(Daudin)*. Amsterdam: North-Holland Publishing Company.

Nishita, M., Hashimoto, M. K., Ogata, S., Laurent, M. N., Ueno, N., Shibuya, H. and Cho, K. W. (2000). Interaction between Wnt and TGF-β signalling pathways during formation of Spemann's organizer. *Nature* **403**, 781-785.

Nordstrom, U., Jessell, T. and Edlund, T. (2002). Progressive induction of caudal neural character by graded Wnt signaling. *Nat. Neurosci.* **5**, 525-532.

Re'em-Kalma, Y., Lamb, T. and Frank, D. (1995). Competition between noggin and bone morphogenetic protein 4 activities may regulate dorsalization during *Xenopus* development. *Proc. Natl. Acad. Sci. USA* **92**, 12141-12145.

Ribisi, S., Jr, Mariani, F. V., Aamar, E., Lamb, T. M., Frank, D. and Harland, R. M. (2000). Ras-mediated FGF signaling is required for the formation of posterior but not anterior neural tissue in *Xenopus laevis*. *Dev. Biol.* **227**, 183-196.

Rieckhof, G. E., Casares, F., Ryoo, H. D., Abu-Shaar, M. and Mann, R. (1997). Nuclear translocation of Extradenticle requires Homothorax, which encodes an Extradenticle-related homeodomain protein. *Cell* **91**, 171-183.

Ruiz i Altaba, A. and Jessell, T. (1991). Retinoic acid modifies the pattern of cell differentiation in the central nervous system of neurula stage *Xenopus* embryos. *Development* **112**, 945-958.

Salzberg, A., Elias, S., Nachaliel, N., Bonstein, L., Henig, C. and Frank, D. (1999). A Meis family protein caudalizes neural cell fates in *Xenopus*. *Mech. Dev.* **80**, 3-13.

Shanmugam, K., Green, N. C., Rambaldi, I., Saragovi, H. U. and Featherstone, M. S. (1999). PBX and MEIS as non-DNA-binding partners in trimeric complexes with HOX proteins. *Mol. Cell. Biol.* **19**, 7577-7588.

Sharpe, C. (1991). Retinoic acid can mimic endogenous signals involved in transformation of the *Xenopus* nervous system. *Neuron* **7**, 239-247.

Shen, W. F., Montgomery, J. C., Rozenfeld, S., Moskow, J. L., Lawrence, H. J., Buchberg, A. M. and Largman, C. (1998). Abd-like Hox proteins stabilize DNA binding by the Meis1 homeodomain proteins. *Mol. Cell. Biol.* **17**, 6448-6458.

Sive, H., Draper, B., Harland, R. and Weintraub, H. (1990). Identification of a retinoic acid sensitive period during primary axis formation in *Xenopus laevis*. *Genes Dev.* **4**, 932-942.

Sokol, S. Y. (1996). Analysis of Dishevelled signalling pathways during *Xenopus* development. *Curr. Biol.* **11**, 1456-1467.

Tada, M. and Smith, J. C. (2000). Xwnt11 is a target of *Xenopus* Brachyury: regulation of gastrulation movements via Dishevelled, but not through the canonical Wnt pathway. *Development* **127**, 2227-2238.

Vlachakis, N., Ellstrom, D. R. and Sagerstrom, C. G. (2000). A novel pbx family member expressed during early zebrafish embryogenesis forms trimeric complexes with Meis3 and Hoxb1b. *Dev. Dyn.* **217**, 109-119.

Vlachakis, N., Choe, S. K. and Sagerstrom, C. G. (2001). Meis3 synergizes with Pbx4 and Hoxb1b in promoting hindbrain fates in the zebrafish. *Development* **128**, 1299-1312.

Wallingford, J. B. and Harland, R. M. (2001). *Xenopus* Dishevelled signaling regulates both neural and mesodermal convergent extension: parallel forces elongating the body axis. *Development* **128**, 2581-2592.

Wallingford, J. B., Rowning, B. A., Vogeli, K. M., Rothbächer, U., Fraser, S. E. and Harland, R. M. (2000). Dishevelled controls cell polarity during *Xenopus* gastrulation. *Nature* **405**, 81-85.

Wallingford, J. B., Fraser, S. E. and Harland, R. M. (2002). Convergent extension: the molecular control of polarized cell movement during embryonic development. *Dev. Cell* **2**, 695-706.

Walshe, J., Maroon, H., McGonnell, I. M., Dickson, C. and Mason, I. (2002). Establishment of hindbrain segmental identity requires signaling by FGF3 and FGF8. *Curr. Biol.* **12**, 1117-1123.

Waskiewicz, A. J., Rikhof, H. A., Hernandez, R. E. and Moens, C. B. (2001). Zebrafish Meis functions to stabilize Pbx proteins and regulate hindbrain patterning. *Development* **128**, 4139-4151.

Whitman, M. and Melton, D. A. (1992). Involvement of p21^{ras} in *Xenopus* mesoderm induction. *Nature* **357**, 252-254.

Wilson, P. A. and Melton, D. A. (1994). Mesodermal patterning by an inducer gradient depends on secondary cell-cell communication. *Curr. Biol.* **4**, 676-686.

Yokota, C., Kofron, M., Zuck, M., Houston, D. W., Isaacs, H., Asashima, M., Wylie, C. C. and Heasman, J. (2003). A novel role for a nodal-related protein; Xnr3 regulates convergent extension movements via the FGF receptor. *Development* **130**, 2199-2212.