Corrigendum

A novel role for a nodal-related protein; Xnr3 regulates convergent extension movements via the FGF receptor

Chika Yokota, Matt Kofron, Mike Zuck, Douglas W. Houston, Harry Isaacs, Makoto Asashima, Chris C. Wylie and Janet Heasman

Development 130, 2199-2212.

On p. 2200, the sequence of Xnr3 morpholino oligo is incorrect. The correct sequence is 5’ TCTCTGGTATTTTGGTG-ACTC 3’.

The authors apologise to readers for this mistake.
A novel role for a nodal-related protein; Xnr3 regulates convergent extension movements via the FGF receptor

Chika Yokota1, Matt Kofron1, Mike Zuck1, Douglas W. Houston1, Harry Isaacs2, Makoto Asashima3, Chris C. Wylie1 and Janet Heasman1,*

1Division of Developmental Biology, Cincinnati Children’s Research Foundation, 3333 Burnet Avenue, Cincinnati, Ohio 45229-3039, USA
2Department of Biology, University of York, York YO10 5YW, UK
3Department of Life Sciences, The University of Tokyo, 3-8-1 Komaba, Meguro-ku, Tokyo 153-8902, Japan
*Author for correspondence (e-mail: heabq9@chmcc.org)

Accepted 6 February 2003

SUMMARY

Convergent extension behaviour is critical for the formation of the vertebrate body axis. In Xenopus, components of the Wnt signaling pathway have been shown to be required for convergent extension movements but the relationship between cell fate and morphogenesis is little understood. We show by loss of function analysis that Xnr3 activates Xbra expression through FGFR1. We show that eFGF activity is not essential in the pathway, and that dishevelled acts downstream of Xnr3 and not in a parallel pathway. We provide evidence for the involvement of the EGF-CFC protein FRL1, and suggest that the pro-domain of Xnr3 may be required for its activity. Since Xnr3 is a direct target of the maternal β-catenin/XTcf3 signaling pathway, it provides the link between the initial, maternally controlled, allocation of cell fate, and the morphogenetic movements of cells derived from the organizer.

Key words: Xnr3, Nodal, Convergent extension, FRL1, FGF receptor, Xenopus laevis

INTRODUCTION

Convergent extension behaviour, broadly defined as the process in which a tissue narrows along one axis and lengthens in a perpendicular axis (Keller et al., 1985; Wallingford et al., 2002), is critical for the formation of the vertebrate body axis. In Xenopus, components of the Wnt signaling pathway have been shown to be required for convergent extension movements but the relationship between cell fate and morphogenesis is little understood. We show by loss of function analysis that Xnr3 activates Xbra expression through FGFR1. We show that eFGF activity is not essential in the pathway, and that dishevelled acts downstream of Xnr3 and not in a parallel pathway. We provide evidence for the involvement of the EGF-CFC protein FRL1, and suggest that the pro-domain of Xnr3 may be required for its activity. Since Xnr3 is a direct target of the maternal β-catenin/XTcf3 signaling pathway, it provides the link between the initial, maternally controlled, allocation of cell fate, and the morphogenetic movements of cells derived from the organizer.

Key words: Xnr3, Nodal, Convergent extension, FRL1, FGF receptor, Xenopus laevis
cysteines (Ezal et al., 2000). No direct tests have so far been carried out on Xnr3 function, but the fact that it was shown to block the mesoderm-inducing activity of BMP4, suggested that it may act by antagonizing BMP signaling (Hansen et al., 1997). A second possible function was suggested by the finger-like protrusions seen in over-expression experiments and by studies using Keller explants of the organizer region, which suggested that Xnr3 may be required for convergent extension movements (Kuhl et al., 2001). The activity of Xnr3 is strongly synergized by co-expression of Xwnt11 mRNA, which is also expressed in the organizer (Kuhl et al., 2001).

Xnr3 is expressed immediately at the mid-blastula transition (MBT) in the dorsal equatorial zone of the blastula and expression becomes highly restricted to the organizer region (Glinka et al., 1996; Smith et al., 1995). This region is responsible for regulating both cell fate and cell movements during gastrulation and neurulation. We have studied the role of Xnr3 using a loss-of-function approach by depleting Xnr3 activity using a moripholino oligo. Xnr3− embryos failed to undergo post-gastrulation dorsal convergent extension movements resulting in embryos with curved axes and split neural folds. The organizer region of Xnr3− embryos showed movements resulting in embryos with curved axes and split neural folds. The organizer region of Xnr3− embryos showed a failure of convergent extension movements that was rescued by non-complementary Xnr3 mRNA. The dorsal mesodermal segment of Xbra expression was missing in Xnr3− embryos at the mid-gastrula stage, and was also missing along the dorsal midline at the neurula stage.

Ectopic over-expression of Xnr3 mRNA in isolated animal caps caused a reciprocal effect; the expression of Xbra, eFGF, NCAM and MyoD and convergent extension movements. We show that the FGF receptor FGFR1 is required for Xnr3-induced elongation movements and expression of Xbra and NCAM, since these effects were inhibited by the antisense depletion of maternal FGFR1 mRNA in animal caps and whole embryos. Furthermore, Xnr3 ectopic expression in animal caps activates MAP kinase, as evidenced by the appearance of phosphorylated ERK protein, showing that Xnr3 activates the FGF signaling pathway. Finally we demonstrate the synergistic interactions of Xnr3 with the FGF receptor EGF-CFC protein, FRL1 and with the cleavage mutant form of Xnr2, cmXnr2. These findings demonstrate the essential role that Xnr3 plays in gastrulation and neurulation, outline the pathway whereby Xnr3 acts and suggest a novel role for nodal family members; that of regulating cell movements through the FGF receptor.

MATERIALS AND METHODS

Oocytes and embryos

Eggs were obtained by injecting Xenopus laevis with 1000 U of chorionic gonadotropin (Sigma Aldrich, Milwaukee, WI), and fertilized using a sperm suspension. Embryos were maintained in 0.1x Marc's Modified Ringer's solution (MMR), and dejellied using 1% thyoglycolic acid (Sigma Aldrich, Milwaukee, WI) at pH 7.8. Staging was according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1956). For injections of morpholino oligonucleotides (MO) or mRNA, embryos were transferred to 2% of Ficoll (Amersham Bioscience, Upsala, Sweden) in 0.3x MMR, and then maintained in 0.1x MMR after the blastula stage. The sites of MO and mRNA injection are described in the text.

For depletion of FGFR1 mRNA, full-grown oocytes were manually defolliculated and cultured in oocyte culture medium (OCM), as described by Xanthos et al. (Xanthos et al., 2002). Oocytes were injected at the vegetal pole with oligos using a Medical Systems picoinjector, in OCM and cultured for a total of 48 hours at 18°C before fertilization. In preparation for fertilization, oocytes were stimulated to mature by the addition of 2 µM progesterone to the OCM and cultured for 12 hours. Oocytes were then colored with vital dyes and fertilized using the host-transfer technique described previously (Xanthos et al., 2002). Three hours after being placed in the frog's body cavity, the eggs were stripped and fertilized along with host eggs using a sperm suspension.

For animal cap assays and Keller explants, embryos were dissected specifically at the late blastula stage using tungsten needles, and maintained in culture on agar in OCM at 18°C. Basic FGF (RD systems; 40 pg/ml) and human activin A (RD systems; 2 pg/ml) were added to the culture medium to treat animal caps during the culture period.

The effects of the morpholino oligo on Keller explants were classified as follows. Class 1: no constriction and no elongation. Class 2: constriction but no significant elongation. Class 3: constriction and elongation. This classification was previously described (Tada and Smith, 2000).

Oligos and mRNAs

A 25mer morpholino oligo (Gene Tools LLC, Philomath, OR) with the following sequence was designed against the Xnr3 5'UTR: 5' GTCTGAAACAGAGCCTCCTCGATTGTGAGTC-3', and the sequence of control oligo with 4 bases altered was: 5' TCAcetyGTTAGATTTGTGGACaGTC-3'.

The MO against eFGF has been described previously and was: 5' ATGGAACAGTCTCCCCAATCAAC-3'.

The sequence of the antisense oligo complementary to FGFR1 receptor was 5' GGGAAAGCGTTCGTGTTGGAGAAGAAG-3' where * indicates a phosphorothioate bond; it was HPLC purified before use (Genosys/Sigma). Oligos were resuspended in sterile, filtered water and injected at 3 or 4 ng into the equatorial region of oocytes. Oocytes were cultured immediately at 18°C.

Capped mRNAs were synthesized using the mMessage mMachine kit (Ambion, Austin, TX), then resuspended in sterile water. Xnr3 ORF was constructed by the overlapping PCR method using the following primers: forward 5'TCGAGACCTCCACAGAGGCT-3'; reverse 5'TCGAATCCATTGATTACATGTCTCCTTGGATACCACTTC-3'.

PCR product was amplified using the Advantage TM-HF PCR kit (BD Biosciences Clontech, Circle Palo Alto, CA), digested with BamHI and Clal, and cloned into CS2+ vector. The template for amplification was pdor3 (Smith et al., 1995). Xdd1 was constructed as described previously (D4) (Rothbacher et al., 2000).

Analysis of gene expression using real-time RT-PCR

Total RNA isolation, cDNA synthesis and real time RT-PCR analysis using a LightCycler System (Roche Molecular Biochemicals, Basel, Switzerland) were performed as described previously (Xanthos et al., 2002). The PCR primer pairs and cycling conditions are listed in Table 1. Omithine decarboxylase (ODC) was used as a loading control, and relative expression amounts were normalized to ODC. Each run had a reverse transcriptase minus sample and a water blank as negative controls.

Whole-mount in situ hybridization

Whole-mount in situ analysis was carried out using DIG-labeled antisense RNA according to the method of Harland (Harland, 1991). Antisense probes were synthesized with the following templates; full-length chd in pBluescript SK(-)/EcoRI digest for chd, full-length Xbra in Psp73/SspI digest for Xbra, and full-length gsc in pBluescript II SK (+)/KpnI.

Immunoblotting

Oocytes or animal caps were lysed in phosphoprotein buffer (80 mM
β-glycerophosphate pH 7.0, 20 mM EGTA, 15 mM MgCl2, 1 mM DTT, 1 mM PMSF, 1:50 protease inhibitor cocktail (Sigma) and cleared by centrifugation at 15,000 × g. The equivalents of 0.5 oocyte or 5 animal caps were loaded on 10% SDS-PAGE Ready Gels (BioRad) and transferred to nitrocellulose. Membranes were blocked in 5% non-fat dry milk (Carnation) in PBS, 0.1% Tween 20 and incubated in primary antibody diluted in the same buffer. Detection was performed using the Super Signal West Pico system (Pierce). Exposure times were approximately 1 minute. Antibodies and dilutions used were anti-diphosphorylated ERK-1 and ERK-2 (1:4000, clone MAPK-YT, Sigma) and anti-α-tubulin (1:10,000, clone DM1A, Sigma).

RESULTS

The phenotype of Xnr3− embryos
To study the function of Xnr3 in early Xenopus embryos we used a morpholino oligo complementary to 25 bases immediately 5' to the start site of the Xnr3 coding sequence (see Materials and Methods). Animal caps over-expressing Xnr3 mRNA undergo elongation movements, so we used this assay to test the specificity of the morpholino. Fig. 1A shows that 100-300 pg of full-length Xnr3 mRNA induced elongation of animal caps, which was blocked by the co-injection of Xnr3 morpholino (MO 20 ng). In contrast Xnr3 morpholino did not inhibit elongation when it was co-injected with Xnr3 mRNA consisting of the open reading frame, lacking the morpholino binding site (ORF mRNA; Fig. 1A).

Xnr3 mRNA is expressed in a highly restricted fashion in the outer epithelial layer of the Spemann organizer over a 4-hour time course, from the late blastula to mid-gastrula stages (Glinka et al., 1996; Smith et al., 1995). To inhibit its activity, we injected the Xnr3 morpholino into the equatorial regions of the two dorsal cells at the 4-cell stage and examined the phenotypes of the injected embryos at gastrula, neurula and tailbud stages (Fig. 1B). Xnr3− embryos had a highly reproducible phenotype.

Blastopore formation was slightly delayed, and the blastoporal ring failed to close over the yolk plug. At the neurula stage, neural folds formed on either side of the open blastopore but failed to fuse and the embryos developed with dorsally
curved and shortened body axes. Increasing doses caused increasing severity of phenotype such that several classes could be recognized (Fig. 1C). The least severely affected embryos closed their blastopores, had closed neural folds, normal heads and slight trunk curvature and shortened axes (class 2 and 3), while more affected cases showed further curvature and shortening together with reduction of head structures (class 4 and 5). Using these categories of defect, we scored the results of using doses of 0-20 ng of morpholino oligo (Table 2). 10 ng of MO caused an average convergent extension phenotype of 3.2 on this scale (87 embryos scored), while 20 ng resulted in an average of 4.2 (61 embryos scored). These data indicate that Xnr3− embryos have a reproducible, dose-dependent gastrulation and neurulation phenotype.

The phenotype of Xnr3-MO-injected embryos strongly resembled that caused by the over-expression of a mutated form of the Wnt pathway component dishevelled (Wallingford and Harland, 2001), which is known to be due to a disruption of convergent extension movements. To study convergent extension movements in the organizer region of Xnr3− embryos specifically, we compared Keller explants dissected from wild-type and Xnr3− early gastrulae. While Keller explants from wild-type embryos showed extensive convergent extension movements (Fig. 1D), explants from Xnr3− embryos showed reduced movement (Fig. 1D,E). To quantify the different degrees of convergent extension movements we classified them into three categories (see Materials and Methods). This data showed that the Xnr3 morpholino reproducibly inhibited

![Fig. 1. Xnr3 MO-injected embryos have gastrulation and convergent extension defects.](image-url)
convergent extension movements in Keller explants (Table 3). To show that the observed phenotype was specific, we rescued the morpholino induced inhibition of convergent extension movements by co-injecting non-complementary Xnr3 mRNA (Fig. 1E and Table 3).

Disruption of gene expression in Xnr3− embryos
Since Xnr3 is a direct target gene of the maternal canonical Wnt pathway known to be essential for organizer formation (Heasman et al., 1994), we asked whether its depletion affected the expression of other organizer genes. We examined by real-time PCR analysis the level of expression of the mRNAs for the transcription factors goosecoid (dorsal mesoderm), Xbra (general mesoderm) and Xvent2 (ventral mesoderm) and of the secreted proteins cerberus and chordin (dorsal mesendoderm) and Xwnt 8 (ventrolateral mesoderm) over the time course of gastrulation. The levels of expression of these genes was little affected by Xnr3 depletion (Fig. 2A and data not shown).

Since neural tube formation in Xnr3− embryos was clearly abnormal, we next examined the effect of Xnr3 depletion on general neural markers NCAM, Xlhbox6 and nrp1 as well as the hindbrain marker engrailed 2 and the anterior neural marker Pax6. Fig. 2B shows that the expression of NCAM was reduced to 10-20% of control levels in Xnr3− embryos, while other general and hindbrain markers were also reduced by higher doses of Xnr3 morpholino.

Since Xnr3 is restricted in its expression to a small segment of the gastrula, we reasoned that the analysis of total expression levels in the whole embryo might not reveal changes in gene expression resulting from Xnr3 depletion in this small area. To address this, we studied the expression pattern of chordin and gsc by whole-mount in situ hybridization of gastrulae and reduced to 10-20% of control levels in Xnr3− embryos, while other general and hindbrain markers were also reduced by higher doses of Xnr3 morpholino.

---

Table 3. Xnr3 MO suppresses elongation movements of Keller explants

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>Average</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uninjected</td>
<td>27</td>
<td>0</td>
<td>0</td>
<td>100</td>
<td>3</td>
</tr>
<tr>
<td>MO</td>
<td>25</td>
<td>28</td>
<td>60</td>
<td>12</td>
<td>1.84</td>
</tr>
<tr>
<td>MO+mRNA</td>
<td>26</td>
<td>0</td>
<td>30.8</td>
<td>69.2</td>
<td>2.69</td>
</tr>
</tbody>
</table>

20 ng of Xnr3 MO was injected into two dorsal blastomeres of the 4-cell stage embryo, and scored after 9 hours as described in Fig. 3. 200 pg of Xnr3ORF mRNA was injected with MO for the rescue.

Fig. 2. Organizer genes are expressed but some neural markers are reduced in Xnr3− embryos. (A) Organizer gene expression was analyzed at gastrula and early neurula stages (stage 10, 10.5, 11, 12, 14). Xnr3 MO does not affect the expression level of organizer genes. (B) Neural markers were analyzed at stage 28 NCAM and en2 expression was repressed in MO-injected embryos. 10 ng (for late markers), or 20 ng (for both early and late markers) was injected into the dorsal marginal two cells at the 4-cell stage. In each case, ornithine decarboxylase (ODC) was used as a loading control (data not shown), and expression of each gene was normalized to the level of ODC expression. (C) chd and gsc are not expressed in the correct region in MO-injected embryos. Expression pattern of chd (top two rows), and gsc (bottom two rows) in uninjected (upper row) and MO-injected (lower row) embryos. chd was expressed normally in MO-injected embryos at stage 11, and continued to be expressed around the dorsal rim of the blastopore even at stage 14. Gsc expression in MO-injected embryos also remained adjacent to the blastopore at stage 14. 20 ng of MO was injected into the two dorsal animal cells of stage 8 albino embryos, and the expression pattern was analyzed by whole-mount in situ hybridization.
neurulae stages (Fig. 2C). *chordin* expression in Xnr3– and wild-type embryos was identical at the mid-gastrula stage, but was in a very different pattern by the early neurula stage (Fig. 2C). In Xnr3– embryos, *chordin* continued to be expressed around the dorsal rim of the blastopore, and to outline only a short notochord in the midline, while in wild-type sibling embryos the expression was found throughout the elongated notochord. Similarly, *goosecoid* expression remained adjacent to the blastopore at the early neurula stage, whereas in control embryos it marked the opposite, anterior pole of the embryo (Fig. 2C). These findings are consistent with the view that Xnr3 is required for convergent extension movements in the midline of the embryo.

**Xnr3 is required for the expression of *Xbra* mRNA specifically in the dorsal mid-line region of the mid-gastrula**

The expression pattern of *Xbra* in Xnr3– embryos at the late gastrula and neurula stages showed interesting changes compared to wild-type embryos (Fig. 3A). At the mid-gastrula stage, *Xbra* mRNA was expressed in an equatorial ring around the blastopore of control embryos, but in Xnr3– embryos the dorsal segment of the ring showed reduced or absent expression of *Xbra* at the mid-gastrula stage (arrow in Fig. 3A). This was a highly reproducible finding (in 14 of 17 cases examined). At the neurula stage, *Xbra* was absent from the notochord of Xnr3– embryos compared to controls (Fig. 3A central panel bottom row).

In these experiments, *lacZ* mRNA was injected together with the morpholino oligo to act as a lineage tracer of the cells in which Xnr3 was depleted. Comparison of the site of Red-gal staining compared to the site in which *Xbra* expression was missing, showed that the cells lacking *Xbra* expression were either the same as or adjacent to those containing the oligo.

The expression of a mRNA coding for a mutated form of the dishevelled protein that lacks the PDZ domain (Xdsh-D4; also called Xdd1) has been shown to cause a similar convergent extension phenotype to that of Xnr3– embryos (Wallingford and Harland, 2001; Wallingford et al., 2000). We next asked whether expression of *Xbra* in embryos over-expressing this dominant negative *dishevelled* mRNA was disrupted in a similar fashion to that in Xnr3– embryos. Fig. 3A shows that the dorsal expression of *Xbra* occurred normally in these embryos at the gastrula stage, but was lost in the midline at the neurula stage in these embryos. Sibling Xdd1 over-

**Fig. 3.** The expression of *Xbra* is absent in the dorsal marginal region of MO-injected embryos at the gastrula stage and from the notochord region at the neurula stage. (A) The expression of *Xbra* in uninjected control (A), Xnr3 MO-injected (B), and Xdd1h mRNA-injected (C) embryos. Dorsal expression of *Xbra* was reduced at stage 11.5 (arrow) and stage 15 in the MO-injected region in Xnr3– embryos (middle column), whereas it occurred normally at stage 11.5 in Xdd1 mRNA-injected embryos (third column). *Xbra* expression in Xdd1 mRNA-injected embryos was lost in the neurula stage (stage 15). (B) Xnr3-induced animal cap elongation is blocked by the expression of dominant negative *dishevelled* mRNA (Xdd1). Xdd1 expression alone does not cause elongation of caps. These samples were frozen and subjected to real-time PCR analysis for the expression of *Xbra*, MyoD, NCAM and nrp1 (Fig. 3B right hand side). All these markers continue to be induced by Xnr3 in animal caps in the presence of Xdd1. (C) The dorsal reduction of *Xbra* expression is specific in Xnr3 MO-injected embryos. The expression of *Xbra* was analyzed in dorsally and ventrally injected embryos. *Xbra* expression in ventrally injected embryos was slightly delayed at the injected region, but was otherwise normal. Arrowheads indicate areas where *Xbra* expression is missing from the blastopore ring. 20 ng of MO was injected together with NLS-*lacZ* RNA into two dorsal or ventral marginal two cells in 8-cell-stage embryos.
expressing embryos went on to develop the convergent extension defect described previously by others (Wallingford and Harland, 2001). This suggests that dishevelled is downstream of Xbra in the dorsal convergent extension pathway. To confirm that dishevelled lies downstream of Xnr3, we tested the ability of animal caps over-expressing Xnr3 mRNA to elongate in the presence of Xdd1. Fig. 3B shows that Xdd1 completely inhibits the ability of Xnr3-injected caps to elongate. Furthermore, Xnr3 overexpressing animal caps, although blocked from elongating by the presence of Xdd1, continue to express Xbra, MyoD and NCAM (Fig. 3B), indicating that dishevelled is downstream of Xbra in the convergent extension pathway.

Next we carried out three tests of the specificity of the Xnr3 morpholino effect on dorsal Xbra expression. Firstly, we reasoned that, since Xnr3 is not expressed laterally or ventrally around the blastopore, injection of Xnr3 morpholino into the ventral side of embryos at the 4-cell stage should not affect the ventral and lateral expression of Xbra. Fig. 3C shows that, apart from causing a slight developmental delay, Xnr3 morpholino had little effect on the ventral and lateral expression of Xbra (3 of 15 cases showed reduced expression). Next, to show that the effect of dorsal Xnr3 morpholino injection was specific for the dorsal Xbra field, we confirmed that the expression pattern of chordin was normal in Xnr3 – embryos (data not shown). Finally we showed that a control morpholino oligo, designed against the same region of Xnr3 but with a four base mismatch, also had no effect on the Xbra expression pattern when injected dorsally or ventrally at the 4-cell stage (data not shown).

These data show that Xnr3 is required to induce the dorsal segment of expression of Xbra mRNA at the gastrula stage, and for the maintenance of its expression in the midline at the neurula stage.

**Over-expression of Xnr3 mRNA in animal caps causes a dose-dependent stimulation of Xbra mRNA expression**

Xnr3 mRNA has been reported to induce the expression of neural markers without stimulating mesodermal gene expression in animal caps (Hansen et al., 1997; Smith et al., 1995). However, in these experiments, Xnr3 mRNA was not tested over a large dose range. Therefore we injected doses of 125 pg-1 ng of Xnr3 mRNA into the animal region of wild-type embryos at the 2-cell stage and dissected animal caps at the late blastula stage. Caps were frozen at sibling mid-gastrula and tailbud stages and sibling caps were examined for elongation movements at the tailbud stage. Increasing doses caused increasing convergent extension movements in animal caps (Fig. 4A), and induced Xbra expression (Fig. 4B). eFGF and eomesodermin mRNA synthesis was also stimulated by Xnr3 expression, and in caps incubated until the tailbud stage, NCAM and MyoD expression was increased (Fig. 4B). In comparison, other mesodermal markers including goosecoid, chordin and cerberus were not activated in animal caps overexpressing these doses of Xnr3 mRNA (data not shown). This is consistent with the view that Xnr3 regulates the expression of specific mesodermal and neural genes.

**Xnr3 functions via the FGFR1 receptor**

The signal transduction pathway downstream of Xnr3 is unknown. However, Xbra is known to be regulated by the FGF/FGFR/MAP kinase signaling pathway (Latekic et al., 1997). Furthermore, MAPK signaling is strongly activated in the dorsal lip during gastrulation (Christen and Slack, 1999; Schohl and Fagotto, 2002). XFD, a dominant negative form of FGFR1, has been shown to block MAPK phosphorylation (Christen and Slack, 1999). Therefore, we next tested whether XFD would block Xnr3 mRNA-mediated activation of Xbra, and NCAM, and of convergent extension movements in animal caps.

In three experiments, co-expression of 500 pg XFD mRNA with 500 pg Xnr3 mRNA in animal caps prevented the convergent extension movements caused by the expression of...
500 pg of Xnr3 mRNA alone (Fig. 5A,B) and prevented Xbra
and eFGF expression at the early gastrula stage (Fig. 5C). Co-
injection of XFD with Xnr3 mRNA also significantly reduced
the expression of MyoD, but did not affect the expression of
the neural marker NCAM (Fig. 5C). Injection of XFD alone
caused no effect on either convergent extension or Xbra and
NCAM expression (data not shown).

If ectopic expression of Xnr3 mRNA in whole embryos
activates an ectopic FGFR/Xbra signaling cascade in embryos
and is responsible for the finger-like protrusions caused by
Xnr3 mRNA expression, then this should be rescued by the co-
injection of XFD mRNA with Xnr3 mRNA. Fig. 5D shows that
there was a significant rescue of the over-expression phenotype
(Xnr3 mRNA alone, 10/10 cases with the over-expression
phenotype; Xnr3+XFD mRNA, 1/10 cases with the
phenotype). This experiment was repeated with a similar result.
This suggests that FGF receptor function is required for Xnr3
activity.

However, XFD inhibits the elongation of animal caps treated
with activin, as well as those treated with FGF (Fig. 5D), and
therefore may be interfering with TGFβ responses through the
activin receptor as well as responses through FGFR (LaBonne
and Whitman, 1994). To test whether FGF receptors, rather
than activin receptors were required for Xnr3 induced
elongation and gene expression, we specifically depleted
maternal FGFR1 since this is the predominant FGF receptor at
the early gastrula stage (Amaya and Kirschner, 1991). We first
demonstrated that an antisense, phosphorothioate-modified, oligo
complementary to FGFR1 depleted maternal FGFR1 mRNA and protein
in oocytes and early embryos, and that zygotic FGFR1 was not
expressed until the end of the gastrula stage (data not shown).
As a test of specificity, we showed that animal caps dissected from FGFR1–
late blastulae were unable to
elongate in the presence of basic
FGF, and this defect was specifically
rescued by the injection of 75 pg of
synthetic FGFR1 mRNA at the 2-
cell stage (Fig. 6A).

Fig. 5. A dominant negative FGFR, XFD, suppresses
phenotypes caused by Xnr3 over-expression. (A)
Morphology of animal caps. Elongation movements
carried by Xnr3 over-expression (Xnr3) was suppressed
by co-injection of XFD (Xnr3+XFD). (B) MyoD and
NCAM expression in animal caps. XFD repressed the
induction of MyoD in Xnr3 over-expressing animal caps.
Xnr3 (500 pg), XFD (500 pg) or Xnr3+XFD (500 pg
each) were injected animaly into two cells of 2-cell-stage
embryos, and animal caps were dissected from stage 9
embryos. Gene expressions were analyzed by real-time
RT-PCR at stage 20. In each case, ornithine
de carboxylase (ODC) was used as a loading control (data
not shown), and expression was normalized to the level of
ODC expression. MyoD expression, but not NCAM
expression was inhibited by coinjection of XFD mRNA
with Xnr3 mRNA. (C) Phenotypes of Xnr3, XFD or
Xnr3+XFD mRNA-injected embryos. XFD rescues both
the head abnormalities and finger-like protrusions of
Xnr3-injected embryos. Embryos were injected with 500
pg of each mRNA at the animal pole (2 cells at the 2-cell
stage). (D) XFD blocks animal cap responses to FGF,
activin and Xnr3. Animal caps from wild-type and
XFD mRNA over-expressing embryos were treated with FGF
(top row) or activin (middle row) as described in
Materials and Methods or co-injected with Xnr3 mRNA.
Caps were dissected at the late blastula stage and cultured
until the late neurula stage. XFD blocked responses to all
three treatments.
Next we tested whether Xnr3 function required FGFR1. Fig. 6B shows that elongation of Xnr3-overexpressing animal caps was inhibited by the depletion of maternal FGF receptor. RT-PCR analysis of these caps showed that the elongation of wild-type caps injected with Xnr3 mRNA coincided with the overexpression of both MyoD and NCAM. In contrast, FGFR1- caps over-expressing Xnr3 mRNA expressed MyoD at the same low level as untreated wild-type animal caps (Fig. 6B). The neural marker NCAM was not affected by FGFR1 depletion (Fig. 6B). Importantly, sibling FGFR1- caps were still able to change shape in response to activin (Fig. 6B), indicating that the activity of the activin receptor was not blocked by FGFR1 depletion, even though Xnr3 was unable to cause elongation. RT-PCR confirmed that activin-treated FGFR1- caps over-

Fig. 6. Xnr3 function in convergent extension requires maternal FGFR1 receptor. (A) Animal caps dissected from FGFR1- late blastulae were unable to elongate in the presence of basic FGF, and this defect was specifically rescued by the injection of 75 pg of synthetic FGFR1 mRNA at the 2-cell stage. (B) Convergent extension movement in Xnr3- overexpressing animal caps was inhibited by the depletion of maternal FGF receptor, FGFR1. FGFR1- caps were also inhibited from responding to basic FGF but not activin. The experiment was repeated with the same result. (C) FGFR1 depletion repressed the induction of MyoD (upper histogram) but not of NCAM (lower histogram) in Xnr3 over-expressing animal caps. In contrast, FGFR1 depletion did not prevent the induction of MyoD by activin. Gene expressions were analyzed by real-time RT-PCR system at stage 20. In each case, ornithine decarboxylase (ODC) was used as a loading control (data not shown), and each bar was normalized to the level of ODC expression. (D) The phenotype of FGFR1- embryos. Oocytes were injected with 3 or 4 ng of antisense FGFR1 oligo, fertilized by the host transfer technique and photographed at the tailbud stage. There was a dose response of gastrulation and convergent extension abnormalities, with dorsally curved axes and open neural folds. (E) Histogram of RT-PCR analyses for Xbra and chordin in sibling embryos of the embryos shown in D, frozen at the early (left histogram) and mid-gastrula (right histogram) stages. FGFR1 depletion (FGFR1- high=4 ng dose of oligo) prevents the expression of Xbra and this was rescued by the re-introduction of FGFR1 mRNA. In contrast, dorsal mesodermal markers such as chordin were little affected by FGFR1 depletion (upper histogram). (F) Xnr3 induces activation of ERK2. Animal caps were isolated from stage 9.5 embryos injected with eFGF (5 pg), FRL1 (2 ng), Xnr3 (500 pg) or Xnr1 (500 pg), cultured until stage 10 and subjected to immunoblotting for phosphorylated (activated) ERK2. α-tubulin was used as a loading control. Oocytes, untreated or incubated in progesterone (+Prog.), were included as negative and positive controls, respectively.
expressed MyoD (Fig. 6C). In contrast to Xnr3-injected FGFR1 caps, no neural induction occurred in activin-treated FGFR1 caps (Fig. 6C). These findings make two important points. Firstly, they argue against Xnr3 acting simply as a weak inducer of the activin receptor, since specific inhibition of FGFR1 leaves activin receptors intact and yet blocks the induction of MyoD by Xnr3. Secondly, they indicate that neural induction downstream of Xnr3 does not require the maternal FGFR1 receptor.

In these experiments, sibling FGFR1−embryos to the animal caps developed with curved body axes and open neural folds (Fig. 6D), a phenotype that is similar to that of Xnr3−embryos (Fig. 1B). A notable difference, however, was that the heads of FGFR1−embryos were more normal than those of Xnr3 MOR-injected embryos.

To find out whether FGFR1 is upstream or downstream of Xbra expression at the early gastrula stage, we examined the expression of Xbra in wild-type, FGFR1−embryos and FGFR1−embryos that were injected with FGFR1 mRNA. Fig. 6E shows that FGFR1 depletion prevents the expression of Xbra and this was rescued by the re-introduction of FGFR1 mRNA. In contrast, dorsal mesodermal markers such as chordin were little affected by FGFR1 depletion (Fig. 6E). This indicates that Xnr3 is upstream of Xbra at the early gastrula stage and that Xnr3 acts via the maternal FGFR1 receptor.

These data suggest that Xnr3 activates the MAP kinase signaling pathway. To confirm this we injected Xnr3 mRNA into animal caps at the late blastula stage and analysed the caps at the early gastrula stage for MAP kinase activity. Western blots were probed with an anti-phospho-ERK antibody. Fig. 6F shows the presence of phosphorylated ERK in animal caps expressing Xnr3 mRNA, but not in uninjected caps. As controls, non-matured and progesterone-stimulated oocytes were included, as well as Xnr1 mRNA and eFGF mRNA animal caps. The doses of mRNA used were those that give robust convergent extension movements (500 pg Xnr3 and Xnr1, 5 pg eFGF). In comparison to Xnr3-induced activation of ERK, eFGF also produced a strong activation, and Xnr1 a weak activation.

Xnr3 activity does not require eFGF but synergizes with the FGFR ligand FRL1

Xnr3 might activate FGFR1 in several ways. Fig. 4B shows that Xnr3 causes the transcription of the growth factor eFGF, which could act as an intermediary between Xnr3 and the FGFR1. We tested this hypothesis by using a morpholino oligo that we have shown previously depletes eFGF in a specific fashion (Fisher et al., 2002). Fig. 7A shows that animal caps depleted of eFGF were able to elongate in the presence of Xnr3 mRNA, and to express Xbra at the gastrula stage. In fact they showed more extensive elongation. Sibling eFGF-depleted embryos developed the expected phenotype and showed reduction of MyoD expression as previously observed (data not shown). This suggests that eFGF may not be a required intermediary in Xnr3-induced convergent extension movements.

Since it is unlikely that Xnr3 activates FGFR1 directly, a second possibility is that Xnr3 acts via another intermediary protein. The EGF-CFC proteins, crypto and one-eyed pin-head have been shown to be essential co-activators of nodal signaling in mouse and zebrafish embryos (Ding et al., 1998; Zhang et al., 1998; Gritsman et al., 1999). FRL1, a distantly related EGF-CFC FGF receptor ligand 1, was first identified in a yeast screen for FGFR binding proteins (Kinosita et al., 1995). It is expressed throughout the Xenopus embryo specifically during the gastrula stage. Over-expression of high doses of FRL1 mRNA induces neural and mesodermal markers and finger-like protrusions similar to those caused by Xnr3 over-expression in whole embryos (Kinosita et al., 1995).

We tested whether FRL1 acted synergistically with Xnr3 in over-expression experiments. Fig. 7B shows that Xnr3 and FRL1 mRNA synergized in animal cap assays to cause excessive elongation. In embryo injection experiments, 500 pg of FRL1 or 50 pg of Xnr3 mRNA injected into 2 ventral cells at the 8-cell stage did not cause finger-like protrusions in whole embryos. Injection of 50 pg Xnr3 mRNA together with 500 pg FRL1 mRNA caused extensive protrusion formation. This data is consistent with the hypothesis that Xnr3 and FRL1 interact to activate the FGF receptor.

An Xnr2 cleavage mutant synergizes with Xnr3 to induce convergent extension movements, Xbra and MyoD

An explanation for the different behaviour of Xnr3 from the other Xnrs may be that the stability and/or activity of Xnr3 protein may depend upon the prodomain, while activation of dorsal mesodermal genes by other Xnrs occurs by the canonical mature protein ALK4/ActRII interaction. Support for this comes from studies with a cleavage mutant form of Xnr2. Uncleaved cmXnr2 was shown to be secreted and active in causing ‘attenuated mesodermal gene expression’, specifically the expression of Xbra in Xenopus early embryos (Eimon and Harland, 2002). We tested whether cmXnr2 acted synergistically with Xnr3. We injected Xnr3 mRNA into animal caps at the 4-cell stage, and then injected cmXnr2 mRNA below the equator into vegetal cells at the 8-cell stage (Fig. 7C). In this way, cmXnr2 could only interact with Xnr3 if it was secreted. Fig. 7C shows that animal caps taken from embryos exposed to secreted cmXnr2 as well as injected with Xnr3 mRNA, elongated significantly more than caps injected with Xnr3 alone. This difference correlated well with a threefold increase in the expression of Xbra in Xnr3+cmXnr2 caps compared with Xnr3-overexpressing caps. There was little change in the basal level of dorsal mesodermal genes in the Xnr3 +cmXnr2 caps, as expected from single injections of cmXnr2 (into vegetal cells) or Xnr3. Secreted cmXnr2 did not cause neural induction, and repressed Xnr3’s ability to induce NCAM expression (Fig. 7C).

This evidence is consistent with the hypothesis that cmXnr2 and Xnr3 act in a similar fashion, requiring the prodomain to activate the convergent extension pathway.

DISCUSSION

The nodal class of TGFβ proteins has been shown to play critical roles in early vertebrate development. They are essential for the establishment of mesodermal and endodermal lineages and for cell movements involved in gastrulation (for a review, see Whitman, 2002). Xenopus embryos are unusual in having an ‘odd man out’ of nodal proteins, Xnr3. Xnr3 does not cause typical axis induction when ectopically expressed, and it does not cause general and dorsal mesodermal induction.
The function of Xnr3 in Xenopus development

in animal cap explants (Smith et al., 1995). It does not rescue VegT-depleted embryos, unlike other nodals (Kofron et al., 1999). In this study, we establish that Xnr3 is both necessary and sufficient for dorsal convergent extension movements in Xenopus embryos. We investigate the signaling pathway of Xnr3 and show that it acts through the FGF receptor FGFR1.

Fig. 7. eFGF depletion, FRL1 and cmXnr2 overexpression increase Xnr3 induced convergent extension activity and gene expression. (A) Animal caps depleted of eFGF with 20 ng of eFGF morpholino (eFGF MO) were able to elongate in the presence of Xnr3 mRNA (right) and to express Xbra at the early gastrula stage (histogram). The experiment was repeated with the same result. (B) FRL1 acts synergistically with Xnr3. 500 pg of Xnr3 mRNA synergized with 500 pg FRL1 mRNA to cause excessive elongation of animal caps (left). RHS shows embryo injection experiments. 500 pg of FRL1 or 50 pg of Xnr3 mRNA injected into 2 ventral cells at the 8-cell stage did not cause finger-like protrusions in whole embryos. Injection of 50 pg Xnr3 mRNA together with 500 pg FRL1 mRNA caused extensive protrusion formation (arrowheads). (C) Animal caps taken from embryos exposed to secreted cmXnr2 as well as injected with Xnr3 mRNA, elongated significantly and showed a synergistic increase in the expression of Xbra and MyoD but not of goosecoid compared with Xnr3-overexpressing caps. Inset diagram shows that Xnr3 mRNA was injected animally at the two-cell stage, and cmXnr2 mRNA was injected into 4 vegetal cells, at the 8-cell stage. Caps were cut at the late blastula stage.
Xnr3 and convergent extension movement

Xnr3 clearly fulfills the description of a molecule that regulates convergent extension movement. Over-expression in animal caps causes the tissue to respond by narrowing along one axis and lengthening in a perpendicular axis. It does this over the same time scale as neural convergent extension occurs in whole embryos. Depletion of Xnr3 in embryos results in dorsal axis curvature and open neural folds, and organizer explants fail to elongate in culture. Convergent extension phenotypes have typically been associated with defects in non-canonical Wnt signaling pathway components (Sokol, 1996; Medina et al., 2000; Habas et al., 2001; Cheyette et al., 2002). The experiments presented here add the novel observation that Xnr3 is an essential activator of this pathway. We show that Xnr3 lies upstream of the Wnt signaling component, dishevelled, since Xdd1 expression in animal caps blocks convergent extension movement caused by Xnr3 ectopic expression. We confirm here that dishevelled is downstream of Xbra expression, since Xbra is expressed normally in Xdd1 mRNA-injected embryos. We also found that Xnr3-induced convergent extension movements in animal caps were inhibited by a morpholino oligo against β catenin, suggesting that canonical Wnt pathway components may also be involved in convergent extension movements (data not shown). One difference we see between Xdd1 over-expression and Xnr3 loss-of-function phenotypes is in head formation. Xnr3− embryos have reduced head structures, unlike Xdd1-expressing embryos. This may be explained by additional effects either of Xnr3 depletion or of Xdd1 expression, over and above their roles in convergent extension. For example, Xdd1 may inhibit canonical Wnt signaling, which is required for posteriorizing the nervous system, resulting in enlarged heads at the expense of posterior tissue (Xanthos et al., 2002). Xnr3 may normally suppress BMP signaling by heterodimerizing with BMP (Hansen et al., 1997); therefore its loss may lead to unaposed ventral signalling causing a reduction in head formation.

Xnr3 and FGFR1

The direct evidence that Xnr3 induces convergent extension activity by activating the tyrosine kinase FGFR receptor FGFR1 is that FGFR1-depleted animal caps over-expressing Xnr3 mRNA are unable to elongate, and that Xnr3 over-expression in animal caps activates the MAP kinase signaling pathway. The expression of Xbra throughout the embryo is greatly delayed by the depletion of maternal FGFR1 mRNA and this is partially rescued by reintroducing FGFR1 mRNA. In these depletion analyses we found that zygotic FGFR1 was not expressed until the neurula stage, which may explain why the depletion of only the maternal FGFR1 component has such a dramatic effect on development (data not shown). Although the dominant negative construct XFD also blocked Xnr3 induced convergent extension movement, it has been shown and was confirmed here to also block activin responsiveness.

The ability of animal caps to express Xbra but not dorsal markers such as gsc and chordin is often described as a ‘weak’ mesodermal response. This work suggests an alternative scenario, that Xbra, in the organizer is not induced by ‘canonical’ Xnr signaling but by signaling of the Xnr3 type through the FGFR receptor. Two pieces of evidence support this idea. Firstly chordin and goosecoid continue to be expressed in FGFR1-depleted embryos, while Xbra is not, and secondly, animal caps depleted of FGFR1 maintain the ability to respond to activin, by elongating and expressing MyoD, while their ability to respond to Xnr3 is interrupted. This study raises the question of whether other Xnrs share the capacity of Xnr3 to activate FGFR.

However, the work presented here does not rule out a role for signaling through activin receptors upstream or downstream of Xnr3 in convergent extension movement. We do not yet have a satisfactory method to test this by loss of function. Indeed we have shown previously that the correct level of Xnr3 expression is dependent on the VegT/nodal signaling pathway as well as being initiated by β catenin/XTcf3 (Xanthos et al., 2002). We do not yet have a satisfactory method to test the role of activin receptors specifically by loss of function in Xenopus.

Xnr3 has also been suggested to act predominantly as a BMP inhibitor, by heterodimerizing with BMPs (Hansen et al., 1997). Although we have not tested this directly, it seems unlikely that this explanation can account for Xnr3 function in convergent extension, since other BMP inhibitors such as noggin and cmBMP7 do not cause elongation when over-expressed in animal caps, nor do they cause Xbra expression (data not shown). We find that inhibition of FGFR1 or XFD function does not block the ability of Xnr3 to activate NCAM expression even though it blocks convergent extension movements. This suggests that neural induction and convergent extension are regulated separately by Xnr3.

How does Xnr3 activate the FGFR receptor? The activity of an intermediary such as eFGF seems unlikely since depletion of eFGF with a morpholino oligo had no effect on Xbra expression or explant elongation. Another interesting possibility is that Xnr3 activation of FGFR depends upon an EGF-CFC protein that was first isolated as an FGFR binding protein, FRL1. Over-expression of FRL1 mRNA causes elongation and NCAM and MyoD expression in animal caps, as well as the formation of finger-like protrusions in whole embryos (Kinoshita et al., 1995). We show that Xnr3 and FRL1 synergize strongly in animal cap assays. Direct tests of FRL1 function and of its possible interactions with nodal proteins are required to determine its role.

A role for the prodomain of Xnrs in activating the convergent extension pathway

TGFβ precursors, consisting of a signal peptide, a large propeptide and a shorter mature region, are covalently linked as homodimers in the N- and C-terminal domains (Gentry et al., 1988). For some family members, the prodomain remains associated after cleavage, and may be responsible for increasing the stability of the mature peptide (Wakefield et al., 1990; Constam and Robertson, 1999). A cleavage mutant form of Xnr2, cmXnr2 is secreted into the culture medium of Xenopus oocytes, and has reduced mesoderm-inducing properties compared to its mature form. It activated Xbra expression in cells distant from the mRNA injection site (Eimon and Harland, 2002). We show that cmXnr2 injected into sites distant from the animal cap synergizes with Xnr3 secreted by the cells of the animal cap, in activating Xbra expression and convergent extension activity. A cleavage mutant form of Xnr3, cmXnr3 was shown to have the same biological activity as Xnr3 itself, suggesting that cleavage is not required for Xnr3 function (Ezal et al., 2000). These
observations raise the possibility that FGFR activation requires the stabilization and/or activity of the prodomain of nodal proteins.

Orthologs of Xnr3 are not present in other vertebrate species. However, the degree to which a nodal proprotein is cleaved into its mature, activin receptor-stimulating form may be regulated in time and space by proprotein convertases (Constand and Robertson, 1999). Nodals may further be limited in their ability to activate specific receptors by the availability of co-ligands or co-receptors such as FRL1 and cripto (Yan et al., 2002). We show here that FGFR1 is the signal transducer for convergent extension movements downstream of Xnr3. It will be important to determine to what extent other nodal proteins share this property with Xnr3.

This work was supported by NIH RO1 HD33002. We would like to thank Dr W. Smith, Dr E. Robertis, Dr J. Smith, Dr U. Rothbacher, Dr A. Zorn and Dr C. LaBonne, Dr M. Pownall and Dr C. Wright for providing reagents, and Dr Rashmi Hegde for useful discussions.

REFERENCES


