Corrigendum

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Development **130**, 2199-2212.

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

The authors apologise to readers for this mistake.

Development 130, 2199-2212
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doi:10.1242/dev.00434

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

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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 βcatenin/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 (Sokol, 1996; Medina et al., 2000; Habas et al., 2001; Cheyette et al., 2002) but the relationship between cell fate and morphogenesis is little understood.

Convergent extension behaviour occurs in both ectodermal and mesodermal cells at the same time as the neural folds and somites form. In mouse, zebrafish and *Xenopus* the T box transcription factor *Brachyury* is required for posterior mesoderm and axis formation (Conlon et al., 1996; Halpern et al., 1993; Herrmann et al., 1990; Schulte-Merker et al., 1994). Xbra has been shown to activate the expression of *Xwnt11* in the *Xenopus* embryo (Tada and Smith, 2000) and Wnt11 in turn regulates convergent extension movements in both zebrafish and frogs (Heisenberg et al., 2000; Tada and Smith, 2000). Fish mutants in *Wnt11* and of the Wnt modulator *glypican 4*, have convergent extension defects without having cell-fate alterations (Heisenberg et al., 2000; Topczewski et al., 2001). Further components of the pathway(s) have been identified in *Xenopus* by dominant negative and loss of function approaches, and include Xfz7 (Medina et al., 2000), strabismus (Park and Moon, 2002), Daam1 (Habas et al., 2001), dapper (Cheyette et al., 2002) and dishevelled (Sokol, 1996; Wallingford and Harland, 2001; Wallingford et al., 2000). Other components include N-terminal c-jun kinase (Yamanaka et al., 2002) and the GTPase RhoA (Habas et al., 2001; Wunnenberg-Stapleton et al., 1999). More recent studies on the JAK/STAT pathway in fish indicate that, rather than one convergent extension pathway, there may be two pathways acting in parallel, STAT3 acting on axial mesodermal cells and Wnt11 in more lateral cells (Yamashita et al., 2002).

Here we show that Xnr3, a member of the nodal sub-class of TGFβ proteins, controls dorsal convergent extension by activating the maternal FGF receptor FGFR1 and regulating *Xbra* expression in the organizer region at the gastrula stage. Xnr3 was first identified in an expression screen for gene products that rescue dorsal development in ventralized *Xenopus* embryos (Smith et al., 1995). It differs from the other five nodal-related genes characterized in *Xenopus* in several respects. *Xnr3* is the only nodal that is a direct target of the maternal Wnt/β catenin pathway (McKendry et al., 1997). While other *Xnrs* are potent axis inducers (Jones et al., 1995; Joseph and Melton, 1997; Takahashi et al., 2000), ectopic expression of Xnr3 leads to the formation of finger-like protrusions (Smith et al., 1995). Structurally, Xnr3 differs from the other Xnrs in lacking the last of the seven conserved cysteines involved in intrachain disulphide bonds, and having a serine instead of glycine located between the second and third

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 morpholino 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 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 FGFR binding 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 0.1× Marc's Modified Ringer's solution (MMR), and dejellied using 1% thioglycolic 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, Uppsala, Sweden) in 0.3× MMR, and then maintained in 0.1× 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′ GTCTGAACAAGAAGCATCTCCTCAGTTGG 3′, and the sequence of control oligo with 4 bases altered was: 5′ TCaCTcGGTAGATTT-GTGGaGAgTC 3′.

The MO against eFGF has been described previously and was: 5′ ATGGAACAGTCATCCCGATCAAC 3′.

The sequence of the antisense oligo complementary to FGFR1 receptor was

5′ G*G*A*CGGTTCGGTTTG*G*A*G 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 mMassage 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′ TCGAGGATCCCCAGAGATGGCAT-TTCTG 3′; reverse 5′ TCGAATCGATTCGATTACATGTCCTTGA-ATCCACATTC 3′.

PCR product was amplified using the Advantage TM-HF PCR kit (BD Biosciences Clontech, Circle Palo Alto, CA), digested with *Bam*HI and *Cla*I, and cloned into CS2+ vector. The template for ampification 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, Switzland) were performed as described previously (Xanthos et al., 2002). The PCR primer pairs and cycling conditions are listed in Table 1. Ornithine decarboxylase (ODC) was used as 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(–)/*Eco*RI digest for *chd*, full-length *Xbra* in *Psp*73/*Ssp*I digest for Xbra, and full-length *gsc* in pBluescript II SK (+)/*Kpn*I.

Immunoblotting

Oocytes or animal caps were lysed in phosphoprotein buffer (80 mM

Table 1. PCR primer pairs and PCR cycling conditions used with the LightCycler™

ααPCR primer pair	Origin	Sequence	Denat. temp.	Annealing temp (°C)/ time (sec)	Extension temp (°C)/ time (sec)	Acquisition temp (°C)/ time (sec)
Xbra	Sun et al., 1999	U: 5-TTC TGA AGG TGA GCA TGT CG-3 D: 5-GTT TGA CTT TGC TAA AAG AGA CAG G-3	95	55/5	72/8	75/3
cerberus	Heasman et al., 2000	U: 5-GCT TGC AAA ACC TTG CCC TT-3 D: 5-CTG ATG GAA CAG AGA TCT TG-3	95	60/5	72/20	81/3
chordin	XMMR	U: 5-AAC TGC CAG GAC TGG ATG GT-3 D: 5-GGC AGG ATT TAG AGT TGC TTC-3	95	55/5	72/12	81/3
eFGF	Casey et al., 1998	U: 5-CTT TCT TTC CAG AGA AAC GAC ACC G-3 D: 5-AAC TCA CGA CTC CAA CTT CCA CTG-3	95	60/5	72/12	83/3
en2	New	U: 5-AAG CAA ACC AAA CAT TTG CC-3 D: 5-ATC CAT AAA CAG GGA AGG GG-3	95	60/5	72/5	80/3
NCAM	New	U: 5-CAC AGT TCC ACC AAA TGC-3 D: 5-GGA ATC AAG CGG TAC AGA-3	95	60/5	72/13	84/3
Xnrp1	Lamb et al., 1995	U: 5-GGG TTT CTT GGA ACA AGC-3 D: 5-ACT GTG CAG GAA CAC AAG-3	95	55/5	72/14	84/3
ODC	Heasman et al., 2000	U: 5-GCC ATT GTG AAG ACT CTC TCC ATT C-3 D: 5-TTC GGG TGA TTC CTT GCC AC-3	95	55/5	72/12	83/3
Pax6	New	U: 5-CGA TGG GCA ACA ATC TAC-3 D: 5-GAC TGA CAC TCC AGG GGA-3	95	55/5	72/10	84/3
XlHbox6	New	U: 5-TAC TTA CGG GCT TGG CTG GA-3 D: 5-AGC GTG TAA CCA GTT GGC TG-3	95	58/5	72/8	87/3
MyoD	Rupp et al.1991	U: 5-AGC TCC AAC TGC TCC GAC GGC ATG AA-3 D: 5-AGG AGA GAA TCC AGT TGA TGG AAA CA-3	95	55/5	72/18	86/3
eomesodermin	New	U: 5-TGG TCC TCA AGG TCA AGT CC-3 D: 5-GGG GAG TTT TCA TTG CTT GA-3	95	56/6	72/10	83/3

β-glycerophosphate pH 7.0, 20 mM EGTA, 15 mM $MgCl_2$, 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 formed but 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

Table 2. Xnr3 MO causes a gastrulation and convergence extension defect in embryo

MO injected (ng)	*n*	MO deficiencies index (%)					Average	Other (%)	Dead (%)
		1	2	3	4	5			
0	96	90.6					1	6.3	3.1
5	73	31.5	6.8	24.7	9.6	8.2	2.5	16.4	2.7
10	87	14.9	5.7	32.2	8.0	20.7	3.2	8.0	10.3
15	61	8.2	13.1	14.8	31.1	16.4	3.4	6.6	9.8
20	63	3.2		7.9	20.6	33.3	4.2	14.3	20.6

Xnr3 MO was injected into two dorsal blastomeres of 4-cell-stage embryo, and scored at stage 30 as described in Fig. 2.

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

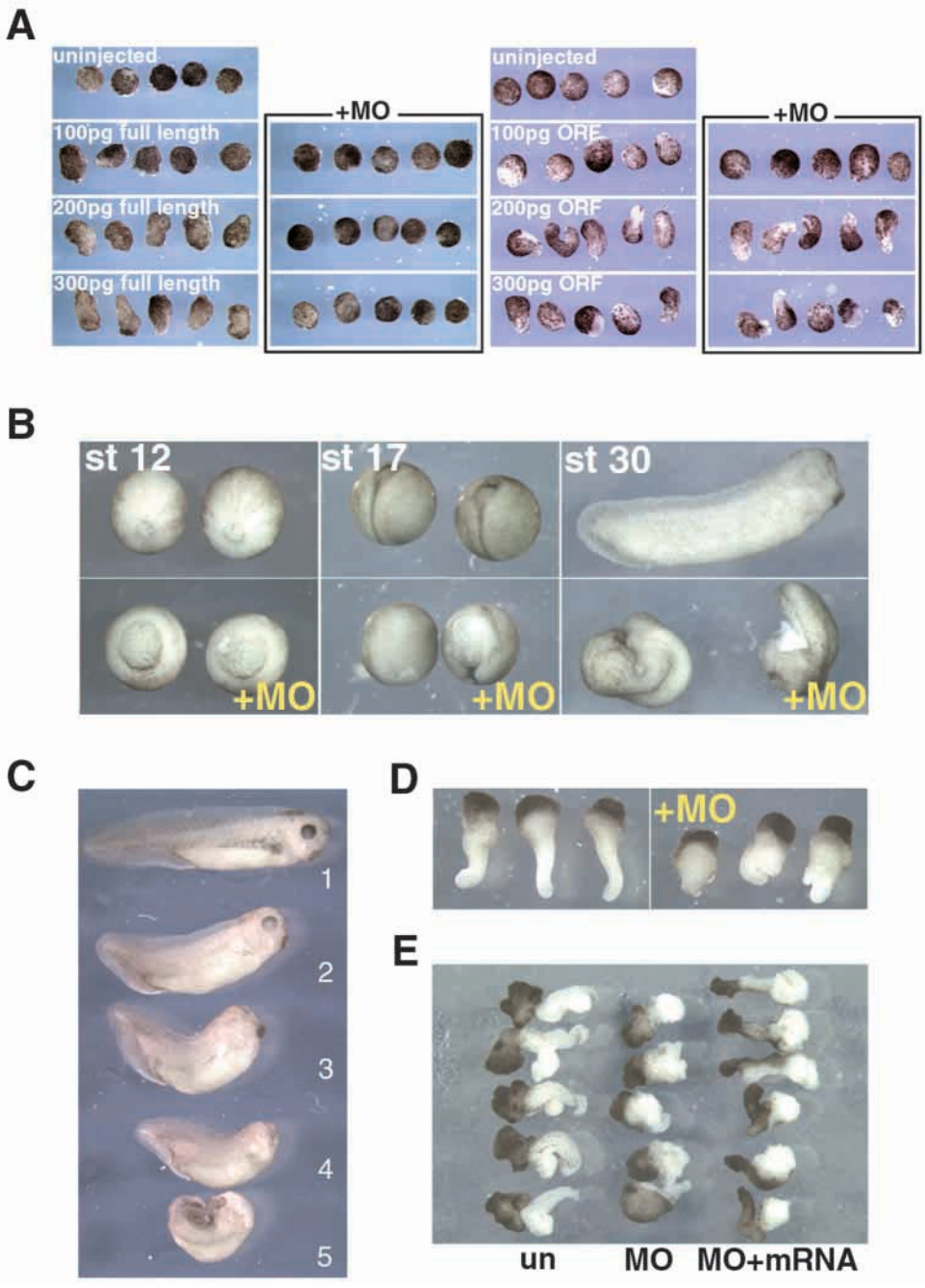

Fig. 1. Xnr3 MO-injected embryos have gastrulation and convergent extension defects. (A) Uninjected animal caps and caps injected with Xnr3 morpholino (MO) were dissected at the late blastula stage and photographed at the late neurula stage (stage 20). The MO blocks elongation movements caused by ectopic expression of full-length *Xnr3* mRNA in the range 100-300 pg (second panel), but not those caused by *Xnr3* ORF (fourth panel). *Xnr3* ORF mRNA consists of the open reading frame only, and lacks the binding site. (B) Sibling, control (top row) and Xnr3 MO-injected (bottom row) embryos at late gastrula (stage 12), neurula (stage 17), and tailbud (stage 30). A total of 20 ng of MO was injected into two dorsal marginal cells at the 4-cell stage. (C) MO injection (0-20 ng) caused a range of defects, scored as five classes of phenotype at stage 38. Numbers on right side of embryos indicate five classes. Class 1: normal embryo. Class 2: embryo has slightly shortened axis. Class 3: closed blastopore and neural fold, normal head and a slightly dorsally curved trunk and shortened axis. Class 4: slightly opened neural folds, dorsally curved trunk and shortened axis. Class 5: open neural folds, dorsally curved trunk and shortened axis. (D) The morphology of Keller explants of uninjected, and 20 ng of Xnr3 MO-injected embryos dissected at sibling stage 10.5 and cultured until sibling stage 24. (E) *Xnr3* ORF mRNA rescues elongation of MO-injected Keller explants. Left column is uninjected explant, middle is MO-injected, and right column is MO + *ORF* mRNA-injected explant. 20 ng of Xnr3 MO and/or 200 pg of Xnr3*ORF* mRNA were injected into the dorsal marginal two cells of the 4-cell-stage embryo and explants were dissected at stage 10.5.

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).

Table 3. Xnr3 MO suppresses elongation movements of Keller explants

		Keller explant elongation deficiencies index (%)			
	n	1	2	3	Average
Uninjected	27	0	0	100	3
MO	25	28	60	12	1.84
MO+mRNA	26	0	30.8	69.2	2.69

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.

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

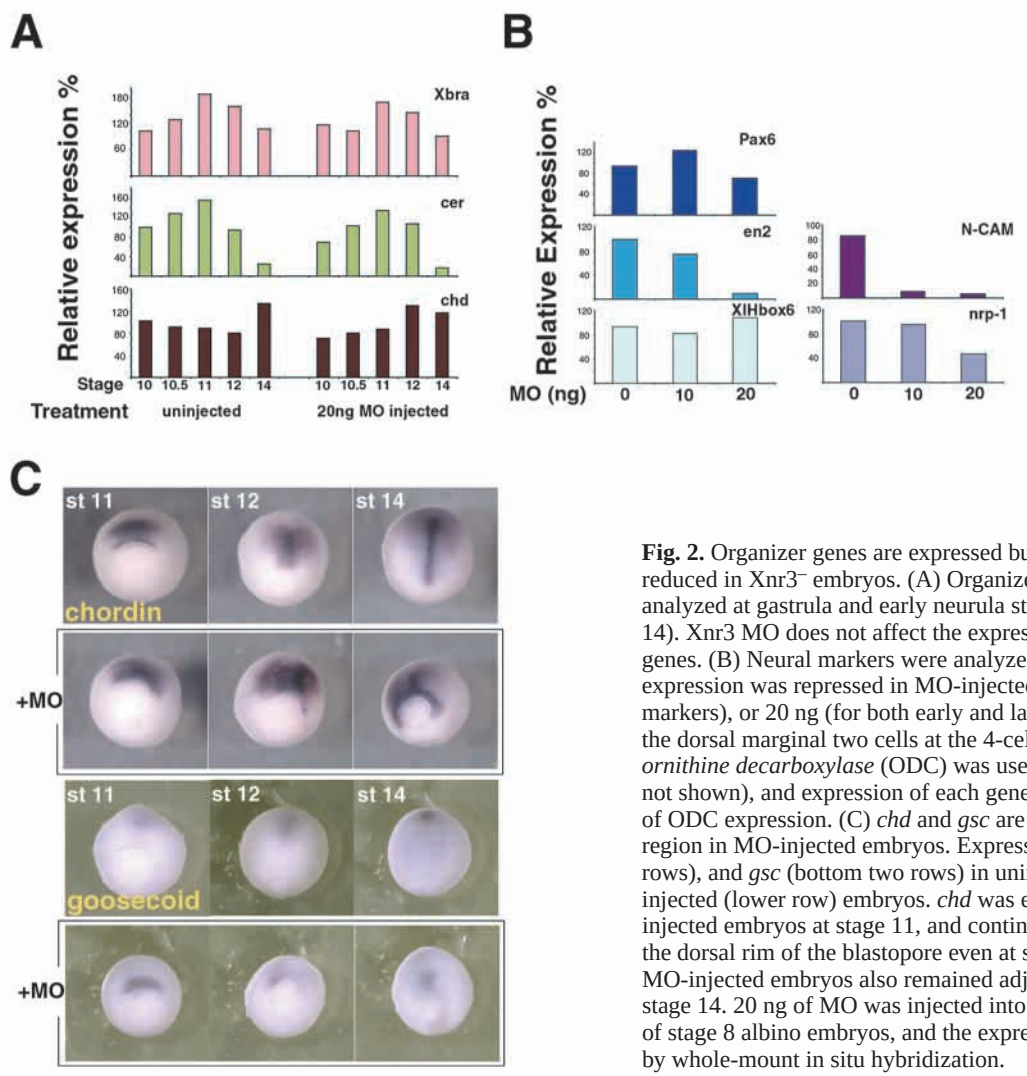

Fig. 2. Organizer genes are expressed but some neural markers are reduced in Xnr3⁻ embryos. (A) Organizer gene expression was analyzed at gastrula and early neurula stages (stage 10, 10.5, 11, 12, 14). Xnr3 MO does not affect the expression level of organizer genes. (B) Neural markers were analyzed at 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-

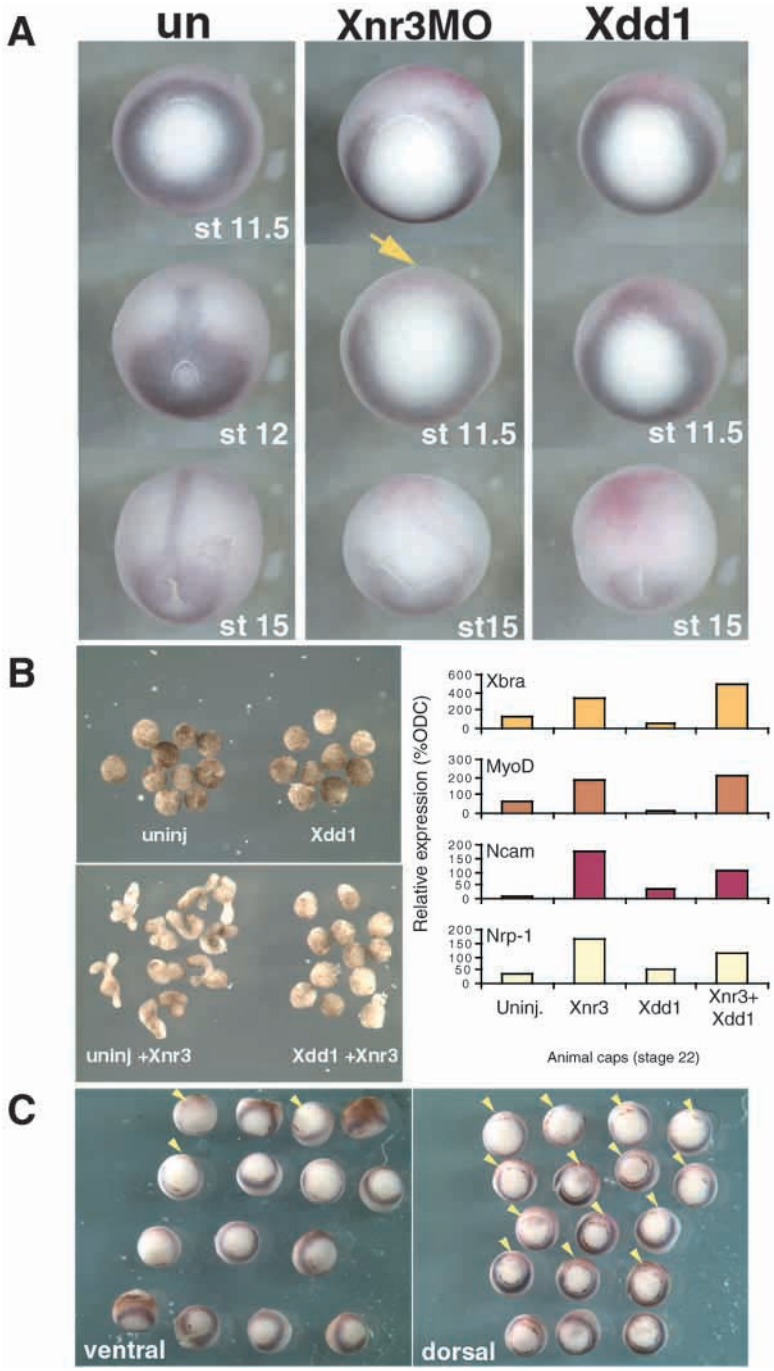

Fig. 3. The expression of *Xbra* is absent in the dorsal marginal region of MO-injected embryos at the gastrula stage and from the notochord region at the neurula stage. (A) The expression of *Xbra* in uninjected control (A), Xnr3 MO-injected (B), and *Xdd1h* mRNA-injected (C) embryos. Dorsal expression 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).

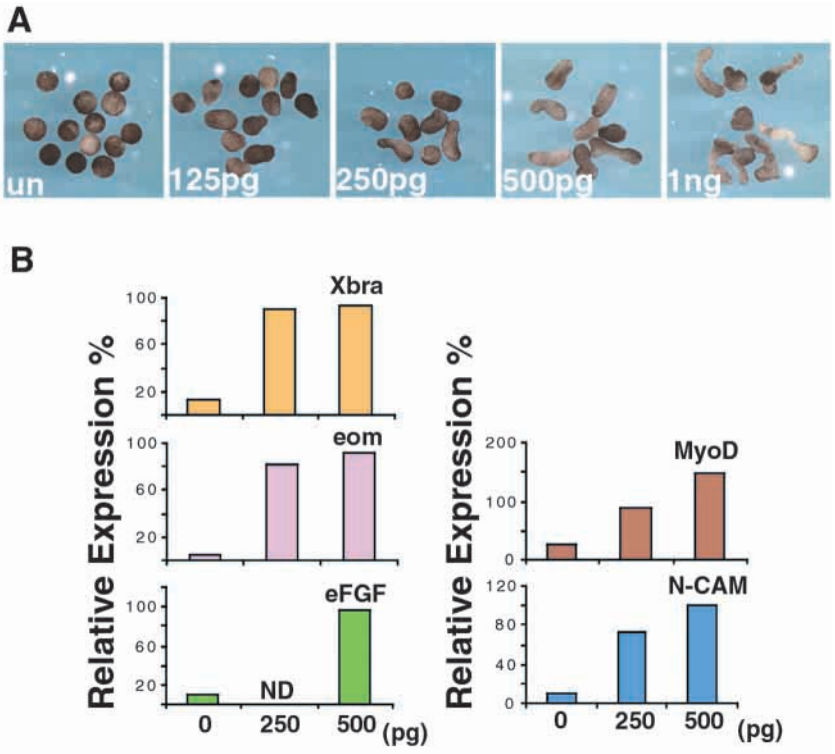

Fig. 4. Over-expression of Xnr3 induces convergent extension movement and the expression of *Xbra*, *eFGF*, *MyoD* and *NCAM* in animal caps. (A) Morphology of animal cap explants injected at the 2-cell stage with a dose response of *Xnr3* mRNA (125 pg-1 ng), dissected at late blastula (stage 9) and photographed at the late neurula stage (stage 20). Elongation is dose responsive. Convergent extension occurs during the neurula stages. (B) *Xbra*, *eFGF*, *eomesodermin*, *MyoD* and *NCAM* expression is induced in animal caps injected with *Xnr3* mRNA. 250 or 500 pg *Xnr3* RNA was injected at the animal pole into two cells of the 2-cell-stage embryo. Animal caps were dissected at stage 9. Gene expressions were analyzed by real-time RT-PCR at stage 11 (for *eomesodermin*, *Xbra* and *eFGF*), and stage 28 (for *NCAM and MyoD*). 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. ND, not done.

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 over-expressing 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 (Latinkic 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).

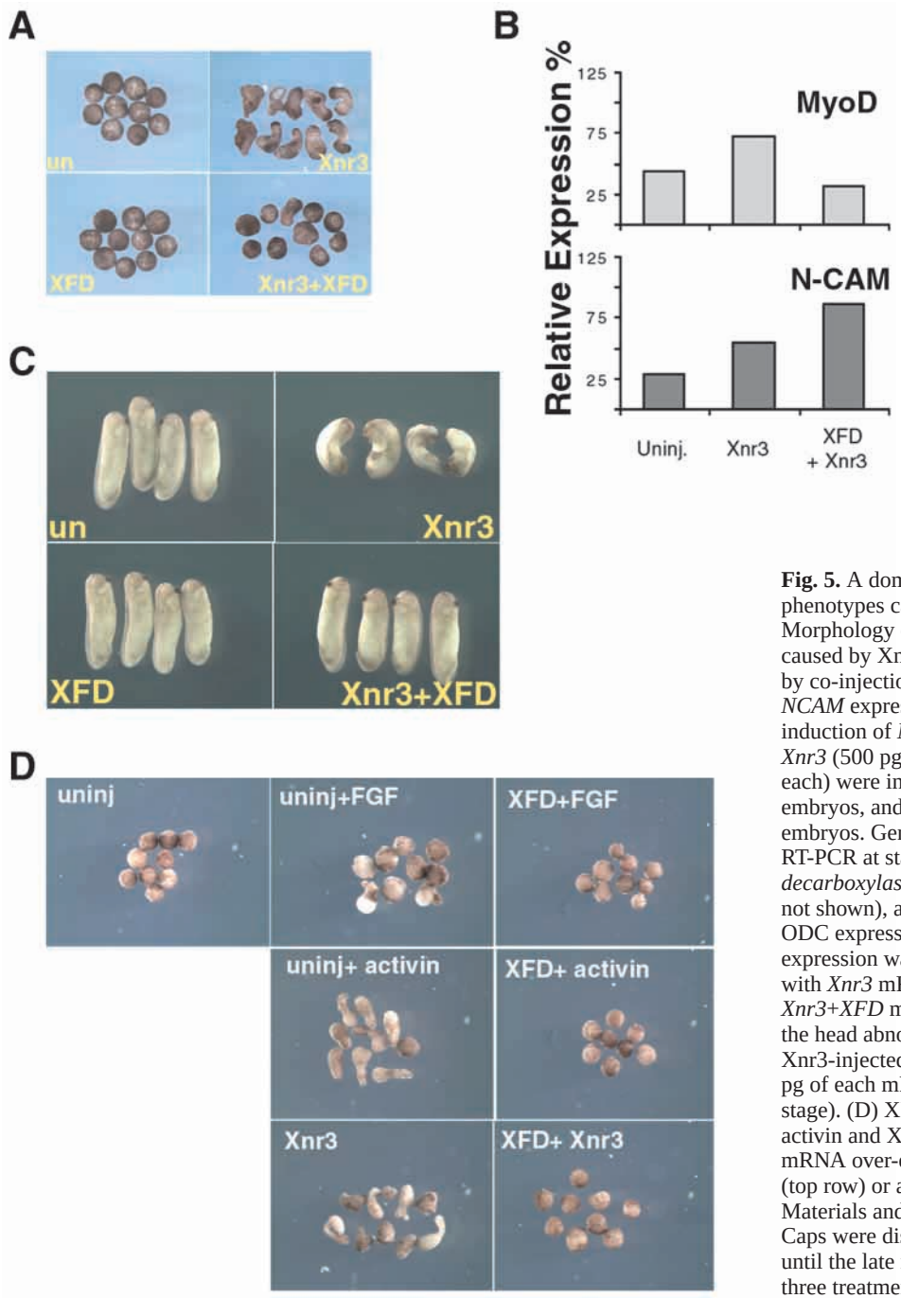

Fig. 5. A dominant negative FGFR, XFD, suppresses phenotypes caused by Xnr3 over-expression. (A) Morphology of animal caps. Elongation movements caused by Xnr3 over-expression (Xnr3) was suppressed by co-injection of XFD (Xnr3+XFD). (B) *MyoD* and *NCAM* expression in animal caps. XFD repressed the induction of *MyoD* in Xnr3 over-expressing animal caps. *Xnr3* (500 pg), *XFD* (500 pg) or *Xnr3+XFD* (500 pg each) were injected animally 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 decarboxylase* (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 over-expression 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-

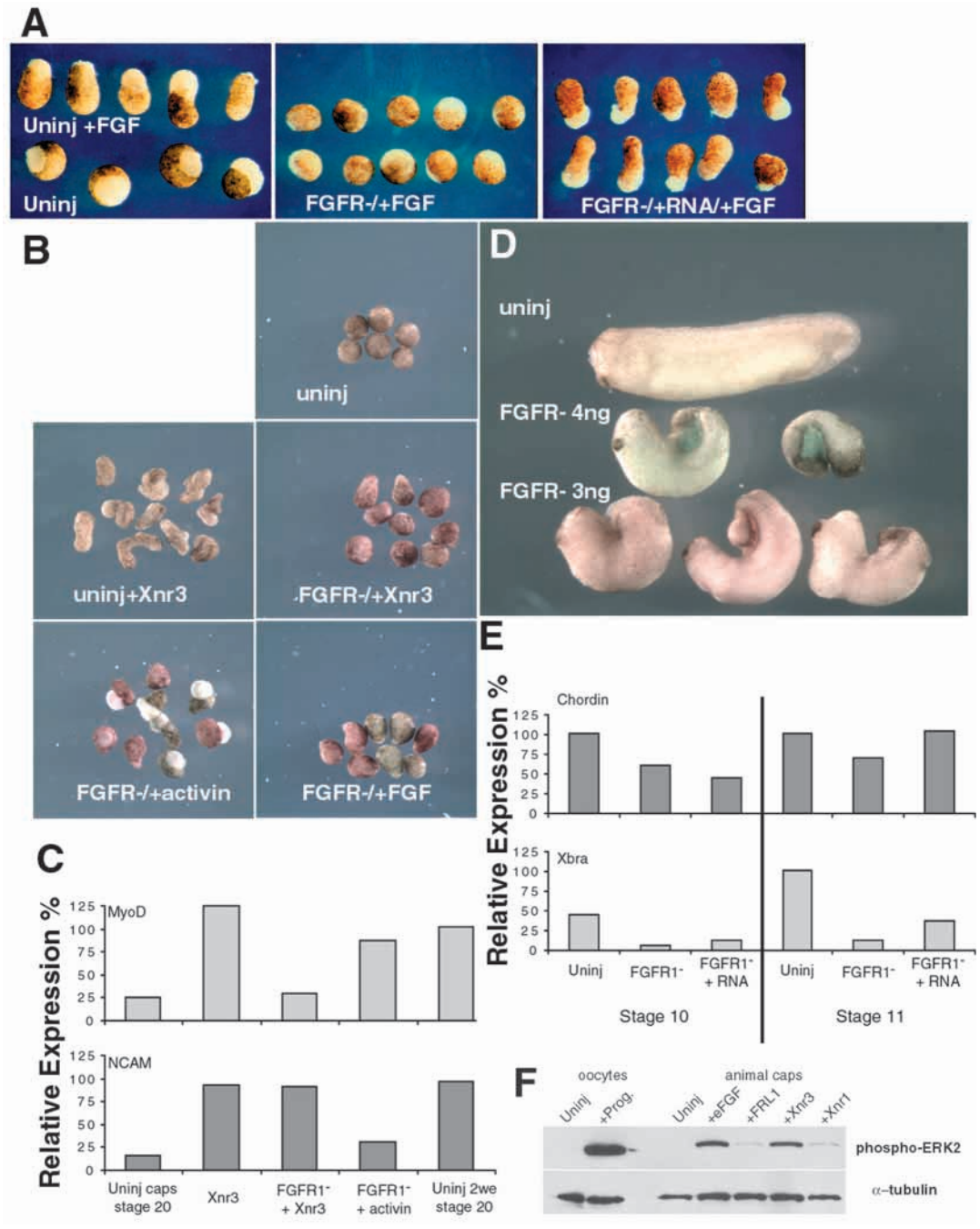

Fig. 6. Xnr3 function in convergent extension requires maternal FGFR1 receptor. (A) Animal caps dissected from FGFR1⁻ late blastulae were unable to elongate in the presence of basic FGF, and this defect was specifically rescued by the injection of 75 pg of synthetic *FGFR1* mRNA at the 2-cell stage. (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 MO-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 FGFR 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 poles of 2-cell-stage embryos, dissected 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 phosophorylated 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 (Kinoshita 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 (Kinoshita 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

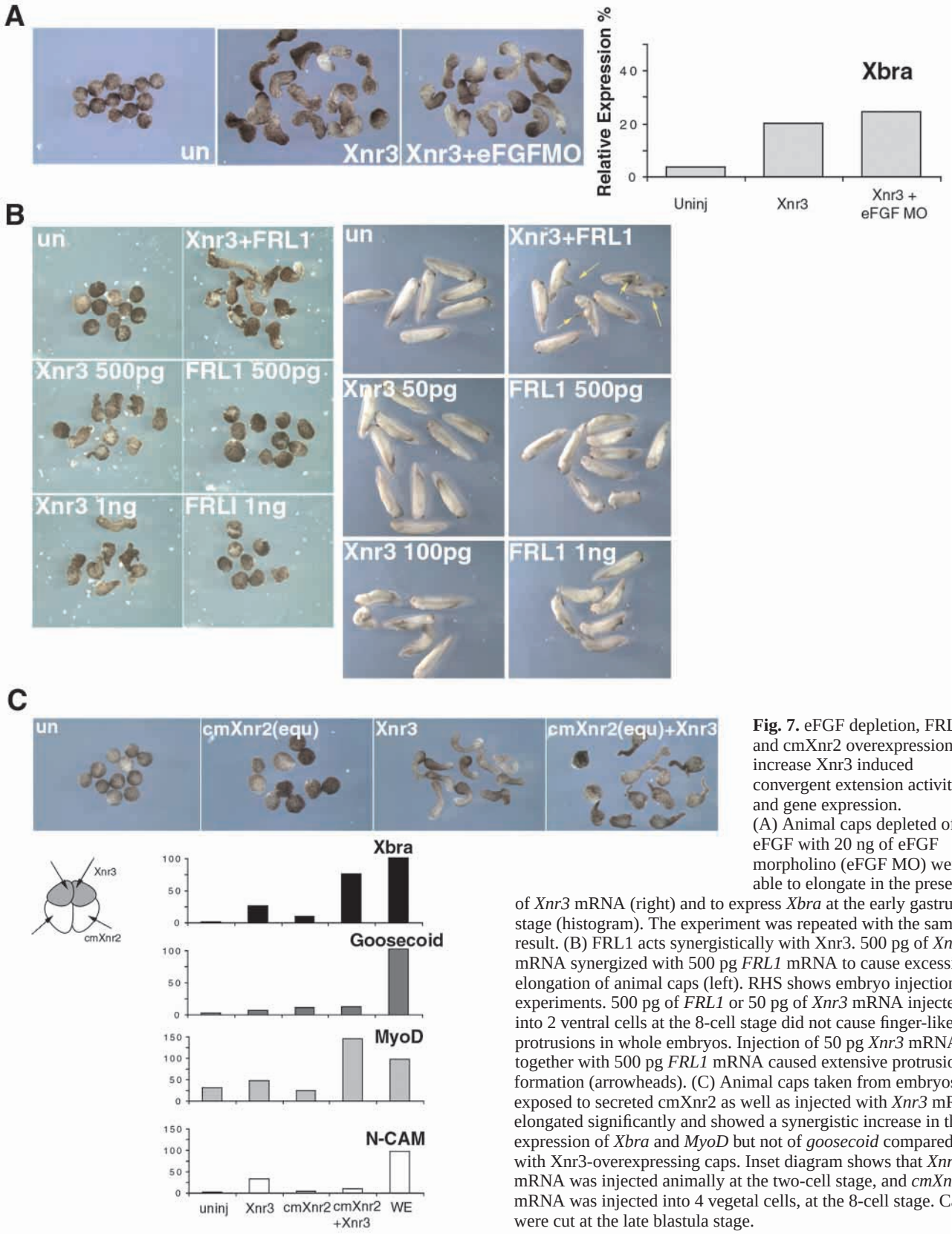

Fig. 7. eFGF depletion, FRL1 and cmXnr2 overexpression increase Xnr3 induced convergent extension activity and gene expression. (A) Animal caps depleted of eFGF with 20 ng of eFGF morpholino (eFGF MO) were able to elongate in the presence of *Xnr3* mRNA (right) and to express *Xbra* at the early gastrula 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.

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.

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 unapposed 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 FGF 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 FGF 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 FGF 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 (Constam 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.

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