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# A novel Cripto-related protein reveals an essential role for EGF-CFCs in Nodal signalling in *Xenopus* embryos

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**Abstract**

The location, timing and intensity of Nodal signalling are all critical for proper patterning of the vertebrate embryo. Genetic evidence from mouse and zebrafish indicates that EGF-CFC family members are essential for Nodal ligands to signal. However, the *Xenopus* EGF-CFC, FRL1, has been implicated in Wnt signalling and in activation of Erk MAP kinase. Here, we identify two additional *Xenopus* EGF-CFCs, XCR2 and XCR3. We have focused on the role of XCR1/FRL1 and XCR3, which are both expressed at gastrula stages when Nodal signalling is active. We demonstrate spatial and temporal regulation of XCR1 protein expression, whereas XCR3 appears to be expressed ubiquitously. Using gain and loss of function approaches, we show that XCR1 and XCR3 are required for Nodal-related ligands to signal during early *Xenopus* development. Moreover, different Nodal-related ligands require different XCRs to signal. When both XCR1 and XCR3 are knocked down, activation of the Nodal intracellular signal transducer, Smad2, is severely inhibited and neither gastrulation nor mesendoderm formation occurs. Together our results indicate that the XCRs are important for modulation of the timing and intensity of Nodal signalling in *Xenopus* embryos.


*Keywords:* EGF-CFC; FRL1; Nodal; Smad2; XCR

## Introduction

Nodal and closely related ligands are members of the Transforming Growth Factor-β (TGF-β) superfamily which play important roles in vertebrate development required for mesoderm and endoderm specification and patterning, for promoting gastrulation movements and establishing the left–right axis (Hill, 2001; Whitman, 2001). Inhibition of Nodal signalling by targeted disruption in mouse, mutation of the two Nodal-related genes *cyclops* and *squint* in zebrafish or use of inhibitors such as Lefty or a truncated derivative of Cerberus in *Xenopus* results in defects in the anterior structures, reduced mesendoderm and defects in the left–right axis (Whitman, 2001). Conversely, ectopic expression of Nodal-related genes in presumptive ectoderm in *Xenopus* induces cells to become mesoderm or endoderm in a dose-dependent fashion (Schier, 2003).

TGF-β ligands signal by binding a type II serine/threonine kinase receptor which then recruits and activates a type I receptor. This in turn phosphorylates and activates intracellular signal transducers, the receptor-regulated Smads (Shi and Massagué, 2003), which, for the Nodal-related ligands, are Smad2 and Smad3. These activated Smads form heteromeric complexes with Smad4, which accumulate in the nucleus and in association with specific transcription factors regulate the transcription of target genes (Shi and Massagué, 2003). In contrast to the one or three Nodal-related ligands in mammals and zebrafish, respectively, there are six Nodal-related ligands (Xnr1-6) in *Xenopus* and a further three functionally related ligands: Derrière, Vg-1 and Activin. All but Xnr3 are thought to induce phosphorylation of Smad2 and Smad3 (Whitman, 2001).

Genetic data from mice and zebrafish indicate that members of the membrane-bound EGF-CFC family are essential for Nodal signalling (Schier, 2003). This family is defined by four founding members: mammalian Cripto and

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Cryptic (Ciccodicola et al., 1989; Dono et al., 1993), zebrafish Oep (Zhang et al., 1998) and *Xenopus* FRL1 (FGF-Related Ligand) (Kinoshita et al., 1995). They have an N-terminal signal sequence, a variant Epidermal Growth Factor (EGF)-like motif, a novel cysteine-rich domain called the CFC domain (for Cripto-FRL1-Cryptic) and a C-terminal hydrophobic region bearing a glycosylphosphatidylinositol (GPI)-anchor signal (Minchiotti et al., 2000). Zebrafish lacking both maternal and zygotic Oep activity phenocopy the double mutant for *squint* and *cyclops* (Gritsman et al., 1999). In mouse, *cripto*, like *nodal*, is necessary for establishing the anterioposterior axis (Whitman, 2001) and targeted disruption of *cryptic* results in defects in left–right asymmetry, a process in which Nodal signalling is also involved (Yan et al., 1999). Biochemical data indicate that Cripto physically interacts with Nodal and ALK4 in 293T cells (Yan et al., 2002), with Xnr1 and ALK4 in *Xenopus* embryos (Harms and Chang, 2003) and with Nodal or Xnr1 in the context of the ALK4/ActRIIB or ALK7/ActRIIB receptor complexes (Cheng et al., 2003; Reissmann et al., 2001; Yeo and Whitman, 2001). This has led to a model in which EGF-CFC family members act as coreceptors required for Nodal to bind the receptor complexes and thus activate downstream Smads.

In contrast to zebrafish and mouse, there is little evidence that the *Xenopus* EGF-CFC family member, FRL1, is linked to Nodal signalling (Gritsman et al., 1999; Tanegashima et al., 2004). Firstly, it was isolated in a yeast screen as a potential FGF-receptor ligand (Kinoshita et al., 1995). Recent work has suggested that FRL1 is involved in neural induction through its ability to inhibit the BMP pathway via the activation of Erk MAP kinase signalling (Yabe et al., 2003). Finally, maternal FRL1 can interact with Wnt11 to activate the canonical Wnt pathway (Tao et al., 2005) and when overexpressed, FRL1 can synergise with Xnr3 (Yokota et al., 2003). Thus, it is thought that FRL1 has a divergent function to the other EGF-CFC family members.

Here, we identify two additional EGF-CFC family members that are expressed during early *Xenopus* development. To reflect their relationship with other EGF-CFC family members, we propose to rename FRL1, XCR1, and to name the two new members, XCR2 and XCR3 (see also Onuma et al., 2005). We have studied in detail the function of XCR1 and XCR3, whose mRNAs are expressed ubiquitously both maternally and zygotically until early neurulation. XCR1 protein expression, in contrast, is temporally and spatially regulated, being primarily expressed during gastrula stages in the animal cap and the marginal zone, but not in the vegetal region. Using both biochemical and embryological approaches and synergy and loss of function experiments, we demonstrate that XCR1 and XCR3 are essential for Nodal signalling in *Xenopus*.

## Materials and methods

### *Cloning of Xenopus EGF-CFC family members and other constructs*

XCR1α was isolated from a stage 10 *Xenopus* cDNA library in λZAPII by PCR and cloned into pFTX4K, a derivative of pFTX5 (Howell and Hill, 1997). The XCR1α sequence is identical to the published sequence (Kinoshita et al., 1995) but for one substitution (C184G) (accession number: AJ864897). Full-length XCR1α was used as a probe to screen the cDNA library, which resulted in the isolation of the XCR1 pseudo-allele (XCR1β, accession number: AJ864898).

*XCR2* (accession number: AJ864899) and *XCR3* were both isolated from the cDNA library by PCR and were cloned into pFTX4K. *XCR3* has two splice forms: *XCR3short* and *XCR3long*, which has an insertion of 72 amino acids after Thr 22 (accession numbers: AJ864900 and AJ864901). The construct UTR-XCR3short contains 45 nucleotides of 5′UTR and the coding sequence of *XCR3short* cloned into pFTX4K.

*Xnrs 4*, *5* and *6* were subcloned into pFTX4K, and the following constructs have been described: *Xnr1*, *Xnr2*, *mActivinβA* and *eFGF* in pSP64T (Jones et al., 1995; Schulte-Merker and Smith, 1995); *Xnr3* and *Derrière* in pCS2+ (Smith et al., 1995; Sun et al., 1999).

### *Embryo manipulations, synthetic mRNA, morpholino injections and in situ hybridisation*

Fertilisation, culture, staging, preparation of synthetic mRNAs for injection and microinjection of *Xenopus* embryos were performed as described (Howell and Hill, 1997). The quantity of synthetic mRNA encoding the Nodal-related ligands injected at the 1-cell stage was the lowest quantity capable of inducing a detectable signal on a Western blot using the antiphosphorylated Smad2 antibody at stage 8 or 10 (for Derrière). The amounts used were: 125 pg of *Xnr1*, 20 pg of *Xnr2*, 1 ng of *Xnr3*, 100 pg of *Xnr4*, 40 pg of *Xnr5*, 500 pg of *Xnr6*, 20 pg of *Activin βA* and 1 ng of *Derrière*. For *XCR1*, *XCR3* and *GFP*, 250 pg of synthetic mRNA was injected and for *eFGF*, 1 ng.

Morpholino oligonucleotides (Gene Tools LLC) designed for XCR1 were: MO XCR1.1, 5′CAAGAAATCTTAAAAACTGCAT3′ (which targets both alleles), MO XCR1.2, 5′AAACTGCATTGTTTTCTGCAAAGGC3′ (which targets XCR1α) (Yabe et al., 2003), and MO XCR1.3, 5′ATTTAATGTGTCCT-CAGCAAAAGCC3′ (which targets XCR1β). For the experiments in Fig. 2, a mixture of MO XCR1.1 and MO XCR1.2 was injected; for the experiments in Fig. 5, a mixture of MO XCR1.2 and MO XCR1.3 was injected. The latter combination does not inhibit the translation of the mRNA synthesised from pFTX4K XCR1α. For XCR3, two morpholinos were designed and injected together: 5′CATGGCACAGTCCTGCTCCAACTAA3′ and 5′CCAGAC-CATGGCACAGTCCTGCTCC3′. The morpholino control was as described (Howell et al., 2002). 40 ng of morpholinos was injected per embryo at the 1-cell stage.

In situ hybridisation was as described (Harland, 1991) with minor modifications. Antisense probes for *Xnot* (Gont et al., 1993), *N-tubulin* (Oschwald et al., 1991) *Xbra*, *Mixer* and *XFKH1* (Howell et al., 2002) were labelled with digoxigenin-UTP (Roche).

### *RNase protection assay*

Isolation of total RNA from *Xenopus* embryos, RNase protection assays and the *EF-1α* and *FGF-R* probes were as described (Howell et al., 1999). Other antisense probes protected nucleotides encoding the following: *XCR1*, amino acids 1–123; *XCR2*, amino acids 1–106; *XCR3*, amino acids 88–161 of *XCR3short* and 160–251 of *XCR3long*, resulting in a single protected fragment.

### *Antibodies, protein extracts and Western blotting*

Peptides corresponding to amino acids 63–79 of XCR1 and 57–73 of XCR3 were used to generate rabbit antisera against XCR1 and XCR3, respectively. IgGs were precipitated from total sera with caprylic acid and affinity purified. Other antibodies used were: antiphosphorylated Smad2, antiphosphorylated Smad1 and anti-Erk (p42/44) (Cell Signalling Technology); antiphosphorylated Erk (clone YT, Sigma); anti-Smad2 and anti-E-cadherin (BD Biosciences) and anti-Smad1 (MADR1, Upstate Technology).

Total cell extracts were prepared by lysing embryos or animal caps in lysis buffer (20 mM Tris pH 8; 2 mM EDTA pH 8; 1 mM EGTA pH 8; 0.5% NP-40; 25 mM β-glycerophosphate; 100 mM NaF; 20 nM Calyculin A; 100 mM pyrophosphate and proteases inhibitors). For XCR1 and XCR3 blots, total protein extracts were treated for 1 h at 37°C with PNGase F (New England Biolabs). The equivalent of one embryo or 10 animal caps was

fractionated on an SDS-polyacrylamide gel. After electrophoresis, proteins were transferred to PDVF membrane (Millipore) and immunoblotted using standard techniques. The antiphosphorylated Smad2 signals were quantified by gel densitometry using ImageJ and normalised to the total amount of Smad2. The fold activation corresponds to the ratio with/without XCR1 overexpression for each ligand whereas the repression corresponds to the ratio of phosphorylated Smad2 when XCR expression is impaired/not impaired by morpholino injection.

## Results

### *Analysis of the temporal expression of XCR1 mRNA and protein*

*XCR1* mRNA is expressed maternally and zygotically until the end of gastrulation (stage 12.5) and then rapidly disappears at early neurula stages (Fig. 1A). The relative expression level of *XCR1* was determined by normalising to the expression of the *FGF-receptor*. The amount of *XCR1* mRNA remains constant until the late blastula stage (stage 9) and then increases 2.5-fold at mid-gastrulation. After which, *XCR1* mRNA starts to disappear and is absent by stage 16 (Fig. 1A).

XCR1 protein was detected using a rabbit polyclonal antibody raised against an XCR1 peptide. To aid visualisation of XCR1, the protein extracts were treated or not with PNGase F, which removes N-linked sugars from glycosylated proteins. The anti-XCR1 antibody recognised the overexpressed protein (Fig. 1B, right panel), which migrates at 26/17 kDa before and after PNGase F treatment, respectively. A similar pattern of bands was observed in extracts from uninjected embryos, demonstrating that the antibody recognises the endogenous protein. XCR1 protein was detected only at very low levels maternally, its expression increased during blastula stages, when levels of mRNA were constant, and peaked at gastrula stages before rapidly disappearing at stage 12 (Fig. 1B). This pattern of protein expression differs from that of *XCR1* mRNA expression and indicates that, at blastula stages, expression of XCR1 must be regulated post-transcriptionally. Because the XCR1 protein expression pattern correlates with the temporal pattern of Smad2 activation in *Xenopus* embryo (Whitman, 2001), we investigated whether it was involved in Nodal signalling.

### *XCR1 synergises with a subset of Nodal-related ligands in early Xenopus embryos*

In *Xenopus*, there are nine Nodal-related ligands, but when this study was done, Vg-1 was thought not to be correctly proessed and active in *Xenopus* embryos (Birsoy et al., 2005) so we did not study it further. For the other eight ligands, we injected 1-cell embryos with a dose of synthetic mRNA sufficient to induce only a low level of Smad2 phosphorylation and asked whether overexpression of XCR1 could potentiate the activity of the ligands. The embryos were harvested at stage 8 when there is no phosphorylation of Smad2 by endogenous ligands (Fig. 2A, UI). XCR1 alone did not induce phosphorylation of Smad2.

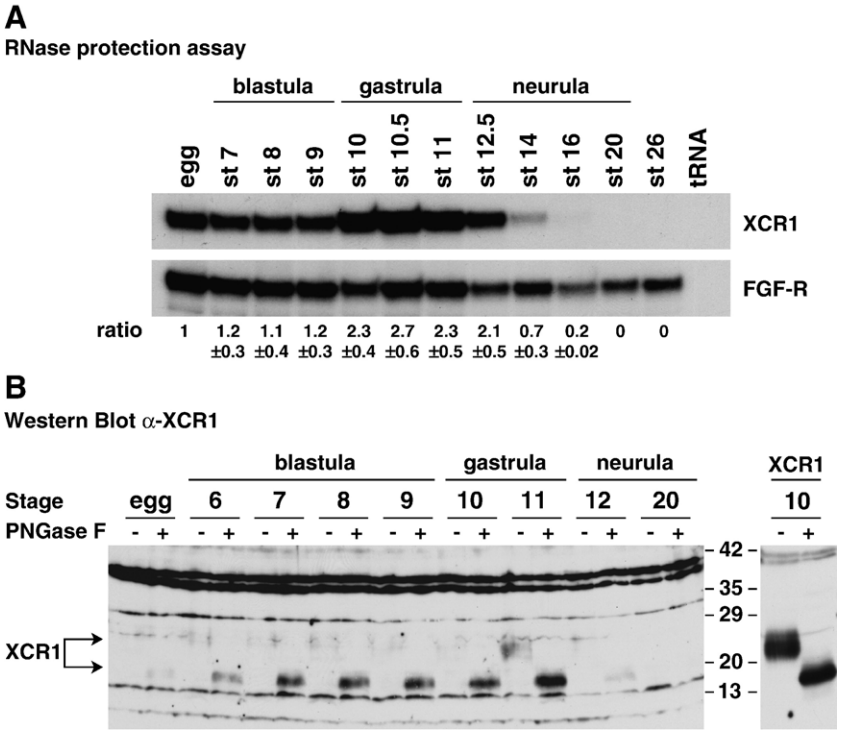


Fig. 1. The temporal expression of XCR1 during embryogenesis. (A) Temporal expression of *XCR1* mRNA in staged embryos assayed by RNase protection. Levels of expression were quantified by Phospho-Imager and normalised to *FGF-R* expression. The results are the mean and standard deviation of three independent experiments. tRNA lane, negative control. (B) The temporal expression of XCR1 protein. Total protein extracts from staged embryos (left panel) or from embryos injected with synthetic mRNA encoding XCR1 (right panel) were treated (+) or not (−) with PNGase F and the expression of XCR1 was detected by Western blot analysis using the anti-XCR1 antibody. The arrows indicate the position of migration of XCR1 ± treatment with PNGase F. The molecular weights (kDa) of markers are indicated.

However, XCR1 potentiated the signalling activity of Xnr1, Xnr2, Xnr5 and Xnr6, but did not synergise with Xnr3, Xnr4, Activin or Derrière (Fig. 2A). Derrière is only active from stage 9 onwards (Lee et al., 2001) but we could not detect any synergy between Derrière and XCR1 even at later stages (data not shown). Xnr3 did not induce phosphorylation of Smad2 at any dose, although it was functional as it induced expression of NCAM in animal caps (data not shown) (Hansen et al., 1997).

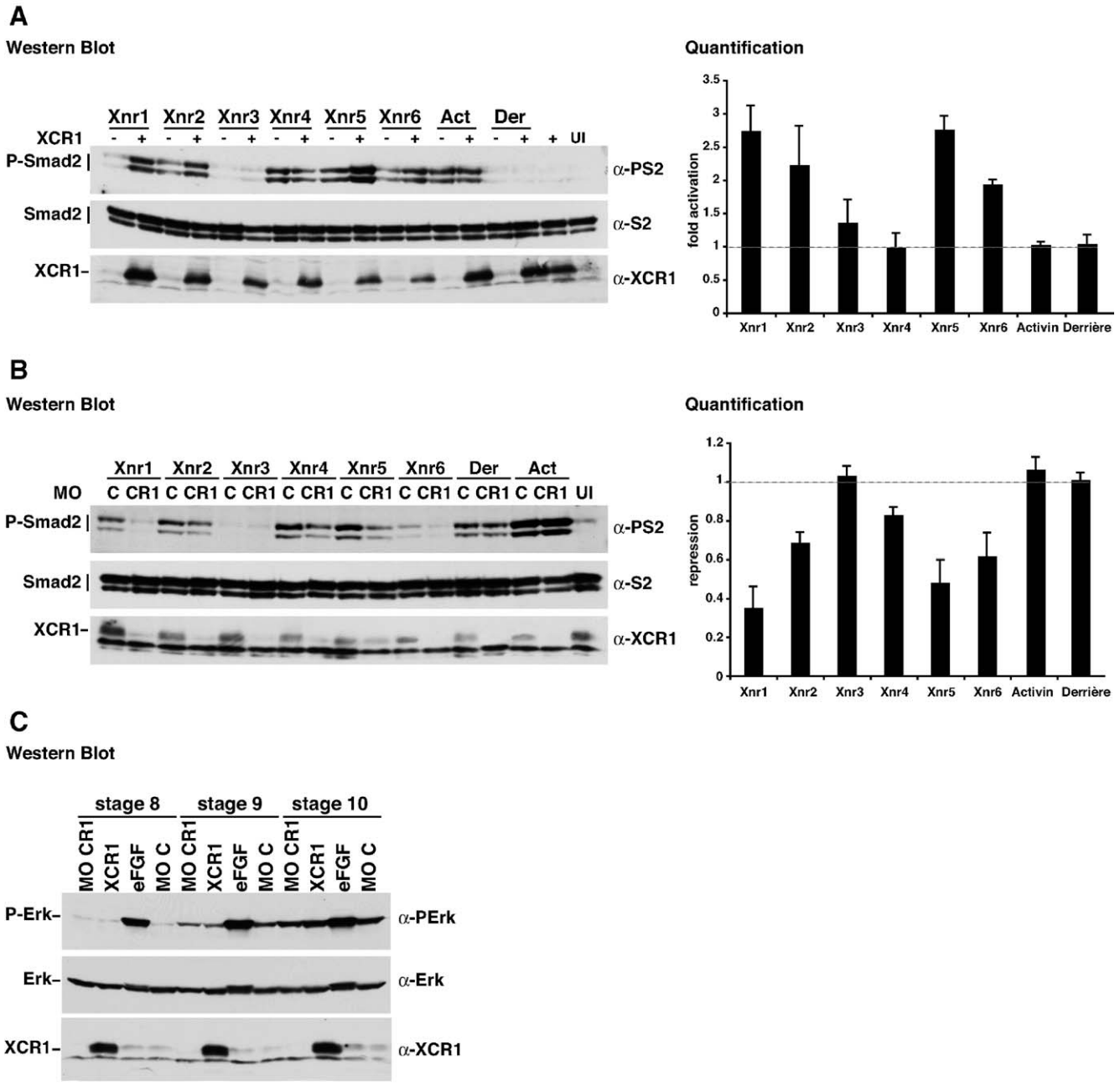


Fig. 2. XCR1 is necessary for signalling by some, but not all Nodal-related ligands (A). Embryos were injected at the 1-cell stage with the indicated synthetic mRNAs; UI denotes uninjected. Der, Derrière; Act, Activin. Embryos were harvested at stage 8 and protein extracts analysed by Western blotting (left panel), detecting activated Smad2 with the antiphosphorylated Smad2 antibody (α-PS2). The membrane was stripped and reprobed with the anti-Smad2 antibody (α-S2) as a loading control. For Smad2 and P-Smad2, the upper band is full-length Smad2 and the lower one is a spliced variant lacking exon 3 (Faure et al., 2000). Expression of injected *XCR1* mRNA was confirmed using the anti-XCR1 antibody. The histogram shows the quantification of three independent experiments (mean ± standard deviation). (B) Embryos were injected at the 1-cell stage with synthetic mRNAs encoding Nodal-related ligands as in panel A together with 40 ng of morpholino control (MO C) or 40 ng of morpholinos against XCR1 (MO CR1). Animal caps were dissected at stage 8 and harvested when sibling embryos reached stage 10. Total protein extracts were analysed by SDS-PAGE and Western blotted with the indicated antibodies (left panel). The histogram shows the quantification of three independent experiments (mean ± standard deviation). In the histograms in panels A and B, the dotted line indicates a level of 1, corresponding to no activation or repression, respectively. (C) Embryos were injected at the 1-cell stage with morpholinos or synthetic mRNA encoding eFGF or XCR1 and harvested at stages 8, 9 or 10. Total protein extracts were analysed by SDS-PAGE and Western blotted for activated Erk (α-PErk, upper panel). Equal loading was confirmed by reprobing the same membrane with an anti-Erk antibody (α-Erk, middle panel) and expression and knockdown of XCR1 were confirmed with the anti-XCR1 antibody (lower panel).

*XCR1 is required for signalling by a subset of Nodal-related ligands in early Xenopus embryos*

Having established that XCR1 specifically synergises with a subset of Nodal-related ligands to activate Smad2, we next investigated the requirement of endogenous XCR1 for signalling by Nodal-related ligands. We designed a combination of morpholinos which targeted both *XCR1* pseudo-alleles. Embryos were injected at the 1-cell stage with synthetic mRNAs encoding the eight different Nodal-related ligands with either the control morpholino (MO C) or morpholinos against XCR1 (MO CR1). At stage 8, animal caps were dissected and cultivated until sibling embryos reached stage 10 and were then analysed by Western blotting using the antiphosphorylated Smad2 antibody (Fig. 2B). The efficacy of the morpholinos was assessed by detecting expression of the endogenous XCR1 protein in embryos injected with MO C or with MO CR1. MO C did not affect the expression of XCR1, but the injection of MO CR1 severely impaired translation of the endogenous XCR1 gene (Fig. 2B, lower panel of blots). The knockdown of endogenous XCR1 by the morpholinos had a significant effect on the ability of Xnr1, Xnr2, Xnr4, Xnr5 and Xnr6 to activate Smad2. Knockdown of XCR1 had no effect on the signalling activity of Derrière, Activin or Xnr3 (Fig. 2B). Similar results were obtained when whole embryos at stage 8 were assayed instead of animal caps (data not shown). This indicates that the effects are direct.

Both gain and loss of function experiments thus show that XCR1 is not involved in signalling by Xnr3, Activin and Derrière, but is required for Xnr1, 2, 4, 5 and 6 to be fully active.

*Loss or gain of function of XCR1 has no effect on the Erk signalling pathway*

Having demonstrated that XCR1 acts upstream of Smad2, we investigated its possible involvement in the Erk pathway, as previous work has implicated XCR1 in this pathway (Kinoshita et al., 1995; Yabe et al., 2003; Yokota et al., 2003). Extracts were prepared from embryos at stages 8, 9 and 10 injected with MO CR1, MO C or synthetic RNA encoding XCR1 or a strong activator of the Erk pathway, eFGF (LaBonne and Whitman, 1997). Activation of the Erk pathway was assessed by Western blotting with an anti-P-Erk antibody (Fig. 2C). Whole embryos were used, as opposed to explants, in order to avoid wounding effects known to activate the Erk MAPK pathway (LaBonne and Whitman, 1997). Overexpression of eFGF potently activated Erk phosphorylation at all three stages. However, neither overexpressing nor knocking down XCR1 had any effect on Erk phosphorylation at any of the stages assayed, suggesting that XCR1 is not involved in Erk signalling.

*XCR1 is expressed in the animal cap and the marginal zone, but not in the vegetal pole of gastrulating embryos*

The Smad2 pathway is activated dynamically in a spatially regulated fashion. Phosphorylated Smad2 first appears in the marginal zone at stage 8.5. By stage 9.5, it is predominantly present in the dorsal marginal zone (DMZ) and in the vegetal pole (VP), then becomes restricted to the ventral/vegetal side of the embryo (Faure et al., 2000; Whitman, 2001).

We investigated whether XCR1 mRNA and protein are expressed in stage 10–10.25 embryos where Smad2 activation is detected. Embryos were dissected into animal caps (AC), marginal zones (MZ), vegetal poles (VP), dorsal and ventral halves (D and V, respectively), using expression of *Sox17α* (predominantly localised to the vegetal pole) (Hudson et al., 1997) and *Xbra* (enriched in the marginal zone) (Smith et al., 1991) as controls for the dissection. *XCR1* mRNA is present throughout the embryo at this stage and when the levels of mRNA were quantitated and normalised to *EF-1α*, the ratio was approximately constant (Fig. 3A, data not shown). We then assessed the regional expression of XCR1 by Western blotting protein extracts from stage 10–10.25 embryos dissected exactly as for the RNase protection assay. XCR1 protein is present both in dorsal and ventral halves at similar levels. It is also present in the animal cap and the marginal zone, but is not detectable in the vegetal pole (Fig. 3B, upper panel). E-cadherin (the loading control) is detectable in all samples (Fig. 3B, lower panel) demonstrating that membrane proteins have been extracted. We compared the expression pattern of XCR1 with the distribution of phosphorylated Smad2 (Fig. 3C). Smad2 is expressed throughout the embryo and is phosphorylated both in the dorsal and ventral halves and in the marginal zone and strongly in the vegetal pole, but not in the animal cap (Fig. 3C).

These data indicate that the pattern of expression of XCR1 protein only partially overlaps with the expression of *XCR1* mRNA, suggesting a spatial regulation of XCR1 expression. We have established above that XCR1 is required for signalling by a subset of Nodal ligands. Since Smad2 is strongly activated in the vegetal region of the embryo in the absence of detectable levels of XCR1, it is unlikely that XCR1 is responsible for regulating Nodal signalling there. However, in the marginal zone, expression of XCR1 does correlate with the presence of phosphorylated Smad2, suggesting a role for XCR1 in Nodal signalling in this region of the embryo.

*Identification of two new EGF-CFC family members expressed in Xenopus embryos*

Since XCR1 can only synergise with a subset of Nodal-related ligands, and Smad2 is activated in a region of the embryo where XCR1 is not detectable, we looked for other EGF-CFC family members in *Xenopus* embryos, and identified two new family members in EST databases, which we have named XCR2 and XCR3 (Fig. 4A). We isolated two isoforms of XCR3 by PCR, XCR3short (XCR3s) and XCR3long (XCR3l), which differ by an additional 72 amino acids in the N-terminal part of the protein. The different EGF-CFC family members share approximately 30% identity overall, but more than 65% identity in the EGF-like domain and more than 40% in the CFC domain. They are no more obviously related to Cripto than to Cryptic (Fig. S1).

*XCR2* mRNA is expressed at an extremely low level maternally. Zygotic expression of *XCR2* starts at stage 12.5 at

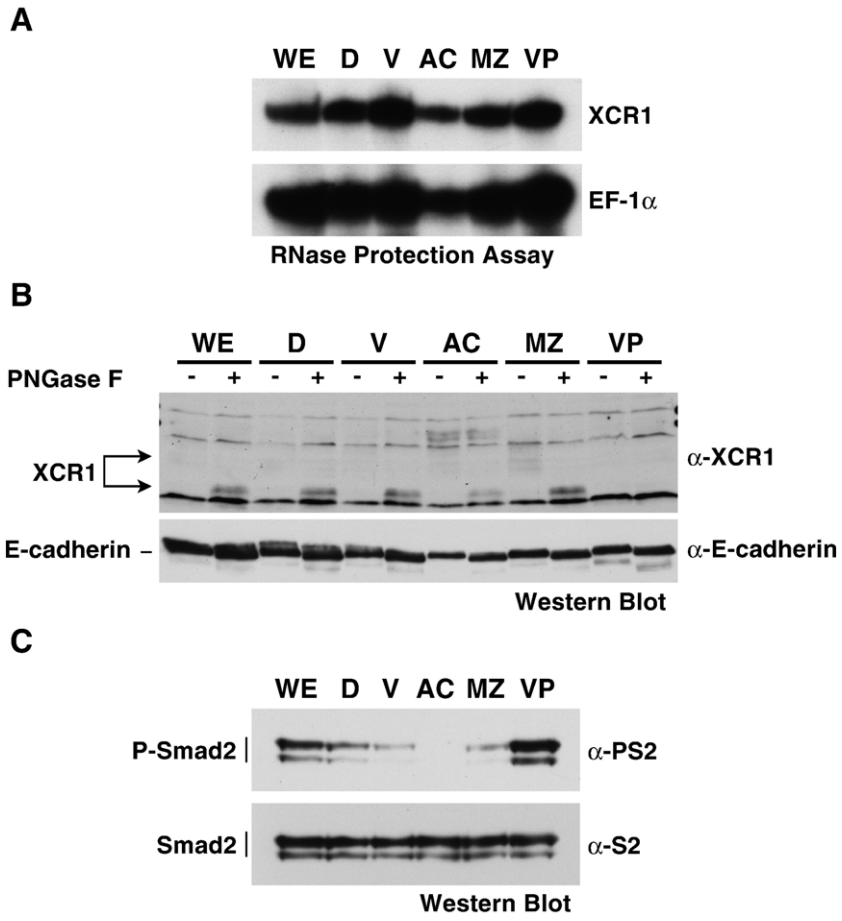


Fig. 3. Spatial expression pattern of XCR1 mRNA and protein. Whole embryos (WE), dorsal halves (D), ventral halves (V), animal caps (AC), marginal zones (MZ) and ventral poles (VP) were harvested at stage 10–10.25 to (A) analyse the expression of *XCR1* mRNA and (B) analyse the expression of XCR1 protein. In panel A, total RNA was purified and *XCR1* expression was assayed by RNase protection assay. *EF-1*α was used as a loading control. In panel B, total protein extracts were treated ± PNGase F prior to analysis by SDS-PAGE. XCR1 protein was detected using the anti-XCR1 antibody. The two arrows indicate the position of XCR1 protein. E-cadherin was used as a loading control. (C) Total protein extracts not treated with PNGase F as in panel B were analysed for the distribution of activated Smad2 by Western blotting using the antiphosphorylated Smad2 antibody (α-PS2) and for the distribution of Smad2 using the anti-Smad2 antibody (α-S2).

the beginning of neurulation, increases until stage 20 and remains constant thereafter (Fig. 4B, upper panel). The pattern of expression of *XCR3* mRNA, in contrast, is similar to that of *XCR1*. Using an RNase protection probe that detects both *XCR3s* and *XCR3l* mRNAs, and normalising to FGF-R expression, we showed that *XCR3* is expressed at a constant level maternally and zygotically until mid-gastrula (stage 10.5; Fig. 4B, middle panel) and then is reduced by 80% by stage 14. The same kinetics were observed using probes specific for *XCR3s* and *XCR3l* (data not shown). As for *XCR1*, *XCR3* mRNA is present throughout the embryo at stage 10–10.25 (Fig. S2).

### *XCR3 is required for signalling by a subset of Nodal-related ligands in early Xenopus embryos*

Since we were primarily interested in the role of EGF-CFC family members in Nodal signalling during gastrulation, we focused on XCR3, which is expressed at this stage. We produced an antibody that recognises both XCR3l and XCR3s. XCR3l migrates at 35/28 kDa and XCR3s at 27/20 kDa before and after PNGase F treatment, respectively (Fig. S3). Unfortunately, we were not able to detect endogenous XCR3 in total *Xenopus* embryo extracts, possibly because the antibody is not sensitive enough or because endogenous XCR3 is present only at low levels. However, we designed morpholinos that could specifically prevent the translation of a synthetic mRNA containing the 5′UTR and the coding sequence of XCR3s (Fig. S3).

We investigated the role of XCR3 in Nodal signalling, performing both synergy and knockdown experiments as we had done for XCR1. We were unable to demonstrate synergy between XCR3 and any of the eight Nodal-related ligands (data not shown), possibly because there is already sufficient XCR3 in the embryo for the ligands to signal efficiently. However, we could demonstrate a requirement for XCR3 for signalling by a subset of Nodal-related ligands in knockdown experiments. Knockdown of XCR3 impaired the ability of Xnr1, Xnr2, Xnr4, Xnr6 and Derrière to activate Smad2, but had no effect on the signalling activity of Xnr3, Xnr5 and Activin (Fig. 4C). The same results were obtained in extracts from stage 8 embryos, suggesting a direct effect of XCR3 knockdown (data not shown).

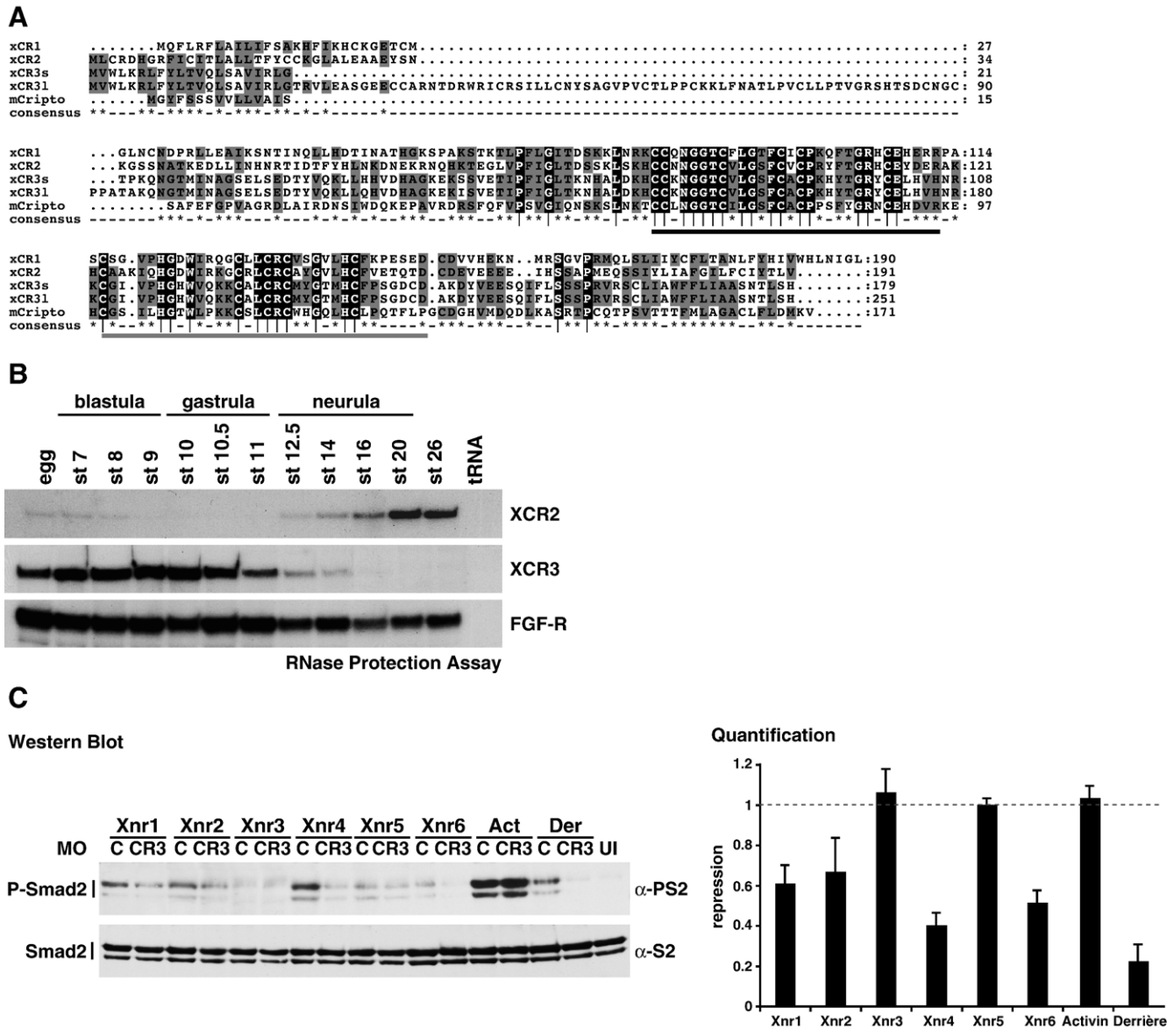


Fig. 4. Characterisation of two new EGF-CFC family members in *Xenopus* embryos. (A) Sequence analysis of XCR2 and XCR3. The sequences of XCR1, XCR2, XCR3 and mCripto were aligned. Conserved residues are marked with (*) and the identical residues with (|). The EGF-like domain and the CFC domain are indicated (black and grey line, respectively). (B) Kinetics of expression of *XCR2* and *XCR3* during *Xenopus* embryogenesis. Total RNA purified from staged *Xenopus* embryos was assayed for the presence of *XCR2*, both splice variants of *XCR3* or the *FGF-Receptor* (*FGF-R*) mRNA as loading control by RNase protection. (C) Embryos were injected at the 1-cell stage with synthetic mRNAs encoding Nodal-related ligands as in Fig. 2 together with 40 ng of morpholino control (MO C) or a combination of the two morpholinos against XCR3 (MO CR3) or were uninjected (UI). Der, Derrière; Act, Activin. Animal caps were dissected at stage 8 and harvested when sibling embryos reached stage 10. Total protein extracts were analysed by SDS-PAGE and Western blotted with the indicated antibodies (left panel). The histogram shows the quantification of three independent experiments (mean ± standard deviation). The dotted line indicates a level of 1, corresponding to no repression.

XCR1 and XCR3 are therefore required for signalling by a partially overlapping subset of ligands. Xnr5 signalling specifically requires XCR1 and signalling by Derrière specifically requires XCR3. Xnr1, Xnr2, Xnr4 and Xnr6 require both XCR1 and XCR3 to signal efficiently.

### *XCR1 and XCR3 are necessary for full activation of the Smad2 pathway*

To assess the role of XCR1 and XCR3 on signalling by endogenous Nodal-related ligands, embryos were injected at the 1-cell stage with morpholinos against XCR1 (CR1), morpholinos against XCR3 (CR3), a combination of morpholinos against both XCRs (CR1 + CR3) or the morpholino control (MO C). At stage 9, depletion of XCR1 or XCR3 individually had no effect, but knockdown of both had a small, but reproducible effect on the phosphorylation of Smad2 (Fig. 5A, upper panel). At stage 10, knockdown of XCR3 had a small effect on Smad2 phosphorylation compared to embryos injected with MO C and impairing expression of both XCR1 and XCR3 had a strong effect on the activation of the Smad2 pathway. At stage 10.5, embryos injected with MO CR3 failed to fully

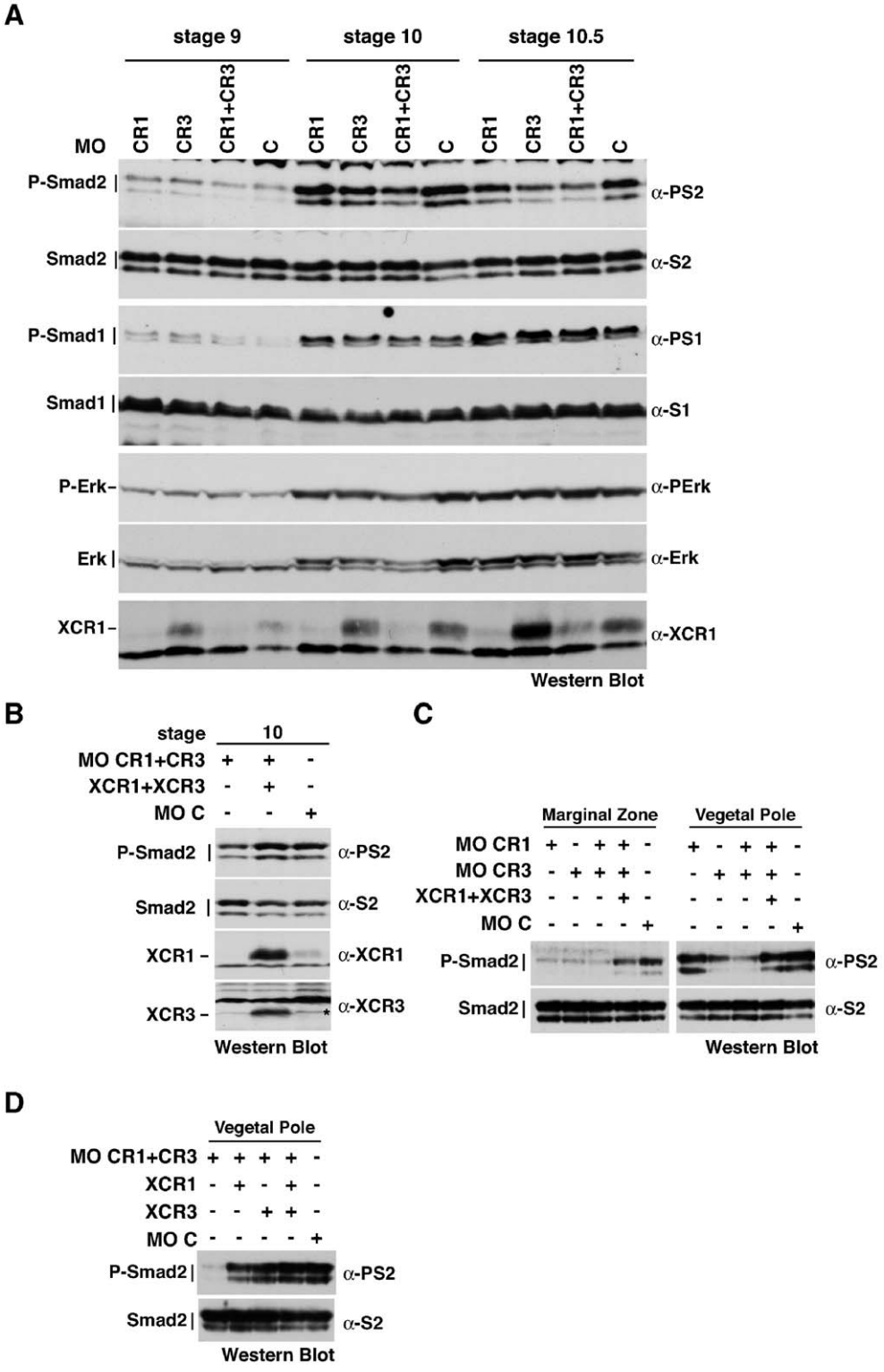


Fig. 5. The role of XCR1 and XCR3 in endogenous signalling by Nodal-related ligands. (A–D) Embryos were injected at the 1-cell stage with a total of 40 ng of the indicated morpholinos alone, or with 250 pg synthetic mRNA encoding XCR1 and/or XCR3 which are resistant to the morpholinos. Embryos were harvested at the indicated stage and total protein extracts were analysed by SDS-PAGE and Western blotted with the indicated antibodies. (A) XCR1 and XCR3 are required for full activation of the Smad2 pathway but not the Smad1 and Erk pathways. (B) Overexpression of XCR1 and XCR3 rescues the effect of the morpholinos. The asterisk indicates a background band that comigrates with overexpressed XCR3. (C) XCR3, but not XCR1, is necessary for Smad2 activation in the vegetal pole. Embryos were injected as above, but also with synthetic mRNA encoding GFP as a tracer. At stage 10, marginal zones and vegetal poles of GFP-positive embryos were dissected and harvested. (D) XCR1 or XCR3 rescues loss of Smad2 phosphorylation in the vegetal pole. Embryos were injected as in panel C and dissected and harvested at stage 10.

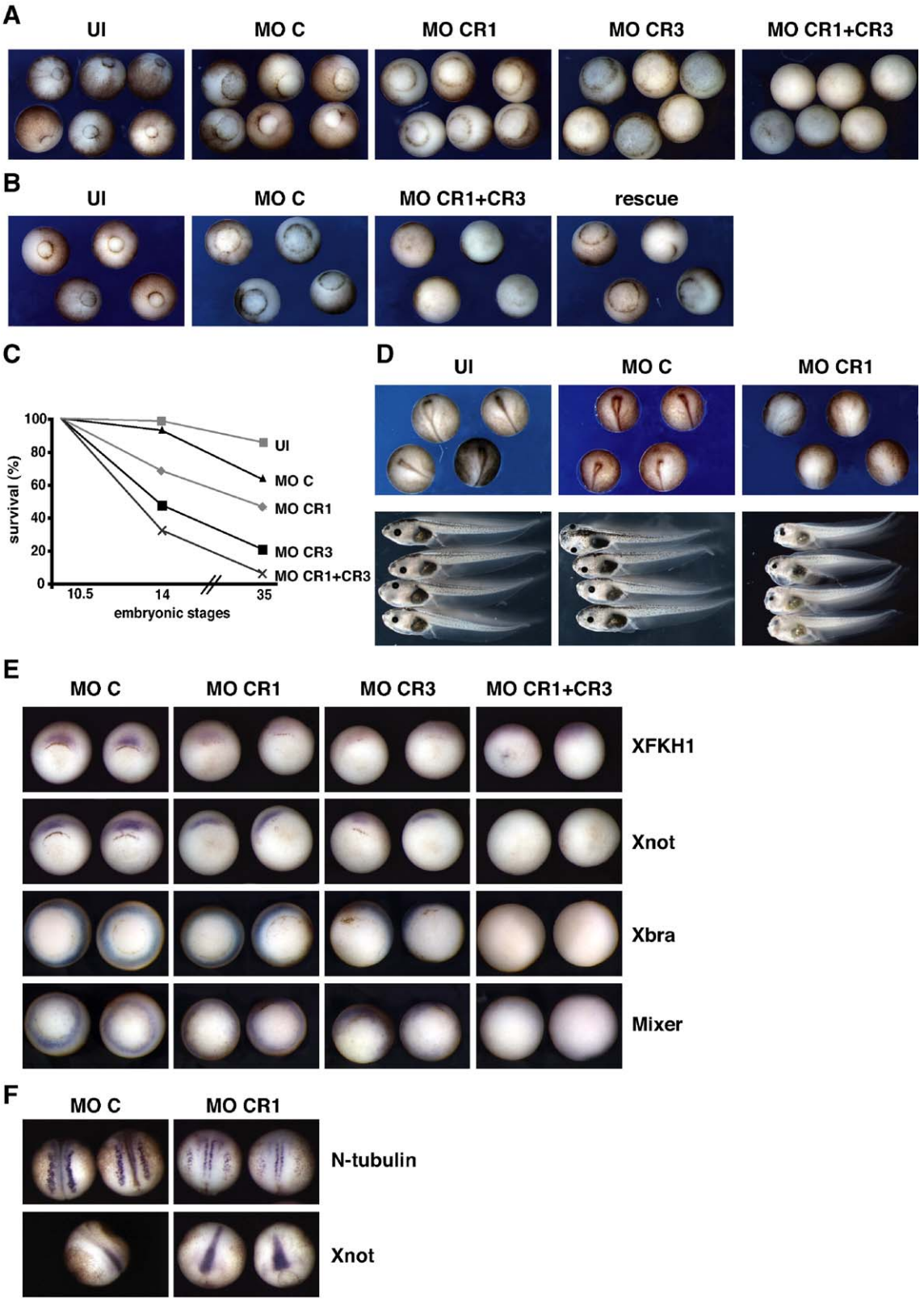


Fig. 6. Phenotype of XCR1 and XCR3 morphants. Embryos were injected at the 1-cell stage with a total of 40 ng of the indicated morpholinos and mRNAs together with GFP mRNA as a tracer. (A) Depletion of XCR1 or XCR3 or both impairs gastrulation. GFP positive embryos were sampled when MO C-injected embryos reached stage 11. (B) Expression of XCR1 and XCR3 rescues the phenotype of embryos depleted for XCR1 and XCR3. (C) Only XCR1 morphants develop further than gastrulation. Embryos were injected as in panel A and scored for survival. (D) Phenotype of XCR1 morphants at neurula stages (stage 16, upper panels) and tailbud stages (stage 45, lower panels). (E and F) Whole-mount in situ hybridisation analysis of the expression of mesendodermal markers (*XFKH1*, *Xnot*, *Xbra* and *Mixer*) or the neuronal marker (*N-tubulin*) at stage 10.5 (E) and stage 16 (F). Pictures show a vegetal view of embryos at stage 10.5 and 11; for stage 16 embryos, dorsal view with anterior up and for tailbud, lateral view.

activate Smad2 to almost the same extent as embryos injected with MO CR1 + CR3 (Fig. 5A, upper panel), suggesting that by stage 10.5 most of the endogenous ligands are XCR3-dependent rather than XCR1-dependent.

We also investigated whether XCR1 and/or XCR3 are involved in the Smad1 or the Erk pathways (Fig. 5A). Injection of morpholinos against XCR1 and XCR3, individually or together, had no effect on the activation of the Smad1 or the Erk pathways, indicating that these EGF-CFC family members are specific for the Smad2 pathway in *Xenopus* embryos. The effects of the morpholinos are specific as coinjection of synthetic mRNAs encoding XCR1 and XCR3 which are resistant to the morpholinos rescued the loss of phosphorylated Smad2 (Fig. 5B).

### *XCR3 is necessary for Smad2 activation in the vegetal region*

We next investigated the contribution of XCR1 and XCR3 to Nodal signalling in different regions of the embryo, in particular assessing whether XCR3 is active in the vegetal region. Embryos were injected as indicated in Fig. 5A together with a synthetic mRNA encoding GFP and we selected only the GFP-positive embryos. At stage 10, knockdown of XCR1 or XCR3 individually or together had a strong effect on the activation of Smad2 in the marginal zone, which could be rescued by the expression of XCR1 and XCR3 (Fig. 5C, left panel). In the vegetal pole, knockdown of XCR1 had no effect on Smad2 phosphorylation as expected, but the injection of MO CR3 substantially decreased Smad2 phosphorylation, showing that XCR3 protein is present and necessary for Nodal signalling in the vegetal pole. This effect was specific as it could be rescued by expression of XCR1 and XCR3 (Fig. 5C, right panel). Interestingly, either XCR1 or XCR3 can rescue the loss of Smad2 phosphorylation in the vegetal pole, suggesting that XCR1-dependent ligand(s) is/are functional in this region, but because XCR1 is not normally expressed there, they might not be fully active (Fig. 5D).

Thus, both XCR1 and XCR3 are involved in endogenous Nodal signalling in gastrulating embryos. XCR1 activity is restricted to the marginal zone, but XCR3 is active both in the marginal zone and the vegetal pole.

### *Downregulation of XCR1 and/or XCR3 expression impairs Xenopus development*

Having established the role of XCR1 and XCR3 in the activation of the Nodal/Smad2 pathway during gastrulation, we then analysed the phenotype of XCR1 and/or XCR3 morphants (Fig. 6). Table 1 summarises three independent experiments.

Depletion of XCR1 alone does not inhibit the formation of the blastopore lip, although it delays its formation. XCR3 morphants in contrast have a much stronger phenotype. The blastopore lip failed to form in 32% of the embryos. In 45% of the XCR3 morphants, the formation of the lip was severely delayed, misplaced and did not close. Embryos depleted of both XCR1 and XCR3 exhibited an even stronger phenotype and 78% of them had no blastopore lip and did not gastrulate. These results suggest that XCR1 does play a role during gastrulation but its function is partially redundant with that of XCR3 at this stage. These effects are specific, as the MO C-injected embryos develop as the uninjected embryos. Moreover, the phenotype of the XCR1 + XCR3 morphants can be rescued by the expression of XCR1 and XCR3 in 85% of the embryos ($n$ = 62, Fig. 6B).

As most of the XCR3 and XCR1 + XCR3 morphants do not gastrulate and subsequently die (Fig. 6C), we only studied the phenotype of XCR1 morphants at later stages. At neurula stages, embryos depleted for XCR1 lacked almost all the anterior neural structures and failed to close their neural tube (Fig. 6D, upper panels). This phenotype is also evident at the tailbud stage when XCR1 morphants have reduced anterior structures (most noticeably the eye) and reduced neural crest

Table 1
Phenotypes of embryos injected with MOs against XCR1 and/or XCR3

| Stage 11 [a] | Wild type (%) | Weak (%) | Medium (%) | Strong (%) |
|---|---|---|---|---|
| MO CR1 ($n$ = 191) | 0 | 52 | 35 | 13 |
| MO CR3 ($n$ = 175) | 0 | 23 | 45 | 32 |
| MO CR1 + CR3 ($n$ = 268) | 0 | 0 | 22 | 78 |
| MO C ($n$ = 166) | 83 | 15 | 0 | 2 |
| UI ($n$ = 240) | 95 | 5 | 0 | 0 |

| Stage 14 [b] | Wild type (%) | Anterior defect (%) | Neural tube closure (%) | Gastrulation defects (%) | Death (%) |
|---|---|---|---|---|---|
| MO CR1 ($n$ = 191) | 4 | 51 | n/a | 13 | 32 |
| MO CR3 ($n$ = 175) | 0 | 17 | 34.5 | 11.5 | 52 |
| MO CR1 + CR3 ($n$ = 268) | 0 | 4.5 | 3 | 26 | 68 |
| MO C ($n$ = 166) | 76.5 | 0 | 0 | 15.5 | 8 |
| UI ($n$ = 240) | 85.5 | 0 | 0 | 8.5 | 6 |

| Stage 45 [c] | Wild type (%) | Anterior defect (%) | Size (%) | Other (%) |
|---|---|---|---|---|
| MO CR1 ($n$ = 89) | 8 | 86 | 64 | 6 |
| MO C ($n$ = 132) | 81 | 6 | 0 | 13 |
| UI ($n$ = 205) | 91 | 0 | 0 | 9 |

[a] Strong, no blastopore; medium, blastopore not closed and lip defects; weak, wider blastopore, embryos developmentally delayed.
[b] Anterior defect, lack of anterior structures and open neural tube; gastrulation defects, failure to close the blastopore; death, embryos degenerated.
[c] Phenotypes for embryos injected with MO CR3 and MO CR1 + CR3 are not included at this stage as most of them do not survive; anterior defect, lack of anterior structures and eyes reduced; size, reduced length compared with uninjected (UI) or MO C embryos.

derivatives. These embryos were overall shorter compared to controls (Fig. 6D, lower panels).

Because XCR1 and XCR3 morphants have gastrulation defects, we investigated the expression patterns of mesendodermal markers by whole-mount in situ hybridisation (Fig. 6E). At mid-gastrula stages, the expression of dorsal mesendodermal markers such as *XFKH1*, *Xnot*, *Xbra* and *Mixer* was reduced in both XCR1 and XCR3 morphants and totally absent in the double morphants. The ability of Xnr1 to induce mesendodermal markers in animal caps was also impaired by knocking down the expression of XCR1 and XCR3 (Fig. S4). At later stages, embryos deficient in XCR1 exhibited a strong neuronal defect, and we found that they showed a downregulation of the neural marker *N-tubulin* (Fig. 6F, upper panels) but not of the posterior mesodermal marker, *Xnot* (Fig. 6F, lower panels). In contrast, the few XCR3 morphants surviving to neurula stages showed a reduced expression of *Xnot*, but not of *N-tubulin* (Fig. S5).

Altogether, these results show that XCR3 is required for the specification of the mesendoderm at gastrula stages, but does not seem to play a role in the neural specification. In contrast, XCR1 is not essential for the gastrulation to occur, possibly because of redundancy with XCR3, but plays an important role in the formation of anterior and neural tissues.

## Discussion

### *The EGF-CFC family in Xenopus embryos*

Here, we show that at least three EGF-CFC family members are expressed during early *Xenopus* development, which are well conserved in the EGF-like and CFC domains. XCR1 is cleaved both at the N- and C-termini (our unpublished data) suggesting that it is GPI-linked and expressed at the cell surface as has been reported for hCripto (Minchiotti et al., 2000). In addition, the change in mobility after PNGase F treatment of endogenous XCR1, as well as all XCRs when overexpressed, demonstrates that these proteins are *N*-glycosylated in *Xenopus* embryos. Human Cripto has also been shown to be *O*-fucosylated on Thr 88 and this is essential for its activity (Schiffer et al., 2001). The fucosylation site is conserved in all three XCRs, but we do not know yet whether *Xenopus* EGF-CFCs are fucosylated.

*XCR1* mRNA is expressed ubiquitously throughout the embryo up until stage 14. However, using an antibody which recognises the endogenous protein, we show that XCR1 is only very weakly expressed maternally. Its expression increases during blastula stages, at a time when mRNA levels are constant, to peak at stage 11. XCR1 protein then disappears rapidly and is not detectable after stage 12. Thus, the protein levels are temporally regulated independently of the mRNA levels. Furthermore, the protein is present only in the animal cap and the marginal zone of stage 10 embryos and is absent from the vegetal pole, whereas the mRNA is ubiquitously expressed. Several possible mechanisms could explain these results. The stability of the protein could be regulated temporally and spatially. We cannot completely rule this out, but believe it is unlikely because upon synthetic mRNA injection, XCR1 is expressed as early as stage 4, indicating that the protein is probably stable at these early stages (our unpublished data). Another possibility that is currently under investigation, is that *XCR1* mRNA is subject to translational control. Indeed, in *Xenopus*, polyadenylation of some mRNAs has been shown to be regulated during development (Simon and Richter, 1994). It is possible that expression of XCR3 is also regulated spatially and temporally, but we have been unable to address this as we could not detect endogenous XCR3 with our antibody.

### *EGF-CFC family members act as coreceptors for Nodal in Xenopus embryos*

Here, we demonstrate that Nodal-related ligands in *Xenopus* embryos require members of the EGF-CFC family to signal. By performing both gain and loss of function experiments, we have shown that the requirement of the different *Xenopus* Nodal-related ligands for EGF-CFCs is not identical. Xnr5 signalling is dependent only on XCR1, Derrière signalling requires only XCR3 and maximal signalling by Xnr1, 2, 4 and 6 requires both XCR1 and XCR3 (Fig. 7A). Xnr3 does not activate Smad2 under any condition tested. We also found that Activin signals independently of XCRs, which is consistent with previous published observations in zebrafish (Gritsman et al., 1999).

Knockdown of both XCR1 and XCR3 strongly reduces the activation of Smad2 by endogenous ligands at late blastula and early gastrula stages, but does not affect the Smad1 or Erk MAPK pathways. This strongly suggests that EGF-CFCs are specific for the Smad2 pathway during *Xenopus* gastrulation. We have investigated the phenotypic consequences of the knockdown of XCR1 and XCR3 during development. At gastrulation stages, XCR1 morphants are delayed but do eventually gastrulate and exhibit defects at neurula stages in the neural tube and anterior structures, consistent with a previous study (Yabe et al., 2003). XCR3 morphants have a much stronger phenotype at gastrula stages with more than 60% of the embryos failing to develop further. Importantly, the double knockdown gives an even more severe phenotype than knocking down either XCR1 or XCR3 individually, with 94% of the embryos either dying before stage 14 or exhibiting severe gastrulation defects (Fig. 6; Table 1). The phenotype of the XCR1 + XCR3 morphant embryos is consistent with the phenotype of VegT-depleted embryos, which do not express Xnr1, 2, 4 or Derrière (Kofron et al., 1999) or embryos injected with Cer-S, a well-characterised Xnr inhibitor (Agius et al., 2000). The fact that the XCR1 + XCR3 morphant embryos have a stronger gastrulation phenotype than the XCR3-depleted embryos indicates that XCR1 also has a role in gastrulation, which is masked by the presence of the ubiquitously expressed XCR3. The presence of XCR3 might explain why this gastrulation role for XCR1 was not previously detected (Yabe et al., 2003). XCR1, in contrast, cannot compensate effectively for loss of XCR3, probably because it is absent from the vegetal pole, where Nodal signalling is high.

It should be noted that knockdown of XCR1 and XCR3 does not completely abolish Smad2 phosphorylation, which could be due to incomplete knockdown of these proteins. Indeed, the effect of morpholinos against XCR1 and/or XCR3 is much

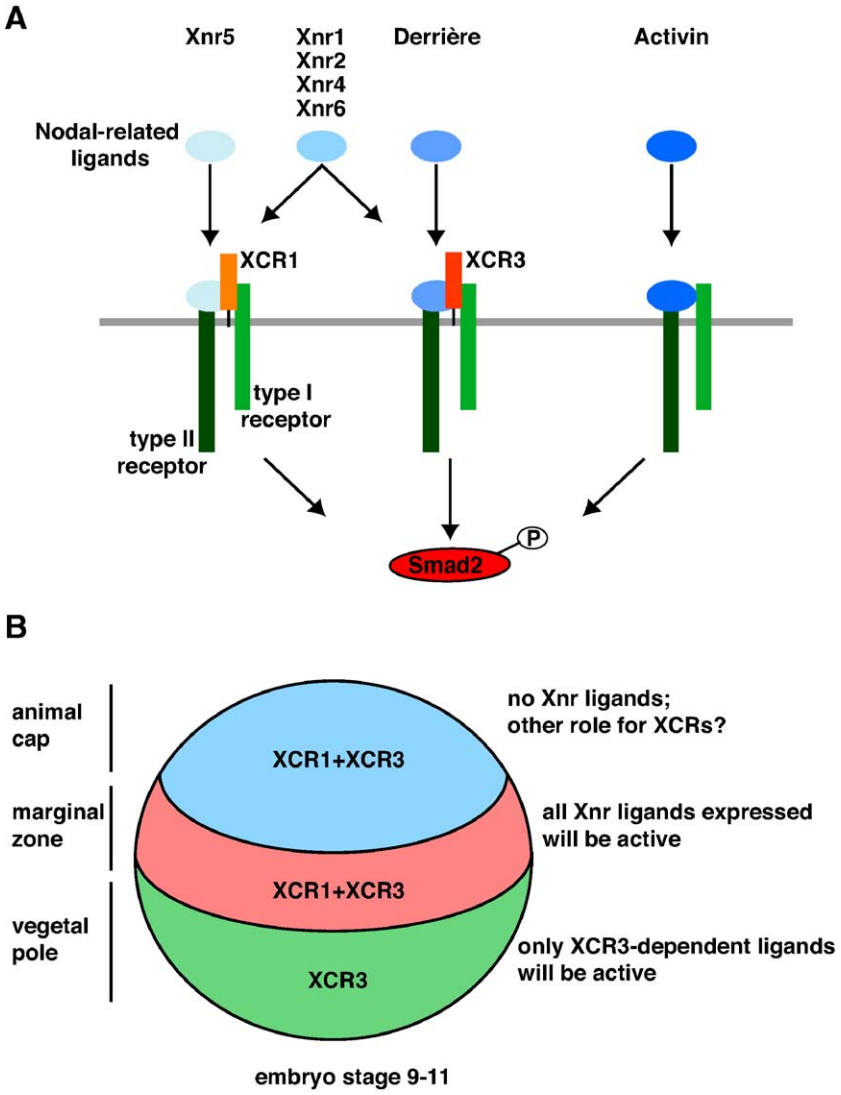


Fig. 7. Schematic model for XCRs function in Nodal signalling during gastrulation. (A) Different Nodal-related ligands require different EGF-CFC to signal. Xnr5 requires XCR1; Derrière requires XCR3. Xnr1, Xnr2, Xnr4 and Xnr6 require both XCR1 or XCR3 to signal efficiently. (B) EGF-CFC expression patterns in the embryo will dictate patterns of Nodal signalling. The vegetal pole is shown in green, the marginal zone in pink and the animal cap in blue. For details, see Discussion.

clearer when GFP is coinjected and only GFP-positive embryos are analysed (compare Figs. 5A and C). Alternatively, it could be due to the presence of yet unknown EGF-CFC members or the presence of endogenous Activin, which has been recently shown to be expressed during early *Xenopus* development (Piepenburg et al., 2004) and whose activity is EGF-CFC-independent.

It was previously suggested that XCR1 synergises with Xnr3 in an animal cap elongation assay, and that this requires signalling through the FGF receptor (Yokota et al., 2003), and it was also reported that XCR1 induces neural genes through the inhibition of the BMP pathway via activation of the MAPK pathway (Kinoshita et al., 1995; Yabe et al., 2003). Although we also observe neural defects in the XCR1 morphants, we were unable to demonstrate any role for XCRs in the Erk MAPK pathway or the BMP pathway, as measured by Smad1 activation, suggesting that the neural phenotype may arise indirectly as a result of a defect in the specification of the organiser in these embryos. Nevertheless, our experiments cannot completely rule out an involvement of the XCRs in other signalling pathways. Maternal XCR1 (FRL1) has also been proposed to be involved in the specification of the dorso-ventral axis through its interaction with Wnt11 (Tao et al., 2005). We have not addressed the role of maternal XCR1, but have focused on the function of the zygotically expressed protein.

In summary, our data clearly demonstrate that *Xenopus* EGF-CFCs are necessary for the different Nodal family members to activate their downstream signalling pathways. Furthermore, we show that XCR1 and XCR3 have different specificities for the different Nodal-related ligands (Fig. 7A).

### *Why are multiple EGF-CFCs expressed at the gastrula stage of Xenopus embryos?*

We have shown that two EGF-CFCs are expressed during the gastrula stages of *Xenopus* embryogenesis which have different specificities for the Nodal-related ligands. Their pattern of

expression is not identical. XCR1 protein is only expressed in the animal cap and marginal zone, whereas our morpholino data suggest that XCR3 protein is expressed throughout the embryo, since knocking it down in animal caps, marginal zones or vegetal poles has a marked effect on either endogenous or exogenous Nodal signalling. Several non-mutually exclusive possibilities can be envisaged to explain why at least two EGF-CFCs are expressed during gastrulation.

The EGF-CFCs could be required for the different Nodal-related ligands to signal through different receptor complexes. Little is known about which specific receptor complexes the different Xnrs bind to, although there is some indication that ALK7 is required for Xnr1 activity, but not for the activity of Xnr2. However, these Xnrs have the same requirement for EGF-CFCs as they require both XCR1 and 3 for activity, which would suggest that, at least for the type I receptors, there is no specificity for a particular EGF-CFC. Alternatively, different EGF-CFCs could confer different signalling properties on the Xnrs. We could not find a good correlation, however, between the ability of a specific ligand to activate a given gene and its dependency on a particular EGF-CFC to signal.

Perhaps the most likely reason for the existence of multiple EGF-CFCs during gastrulation is to modify the spatial and temporal activity of Nodal-related ligands (Fig. 7B). Although we know little about where each Nodal-related protein is expressed, the broad boundaries of expression can be inferred from their mRNA expression patterns. In situ hybridisation indicates that *Xnr5* and *6* are the first to be expressed in the presumptive endoderm from stage 8+ (Takahashi et al., 2000). *Xnr1* and *2* are expressed somewhat later and accumulate in the dorsal marginal zone by stage 10 and *Xnr4* expression is restricted to the deep cells of the organiser at this stage (Jones et al., 1995; Joseph and Melton, 1997). *Derrière* is initially expressed throughout the presumptive mesendoderm and is then restricted to posterior mesendoderm (Sun et al., 1999). Derrière activity requires XCR3, which appears to be ubiquitously expressed. Thus, Derrière is probably active wherever it is expressed. Xnr5, however, which requires XCR1, will only be active in regions where its expression overlaps with that of XCR1. The dependency of Xnr1, 2, 4 and 6 on both XCR1 and XCR3 for their full activity will limit the maximal activity of these ligands to regions of the embryo where both XCRs and the ligands are expressed. These ideas will be more systematically tested in the future when it is possible to determine exactly where the different ligands are expressed.

In summary, we show here that at least two EFC-CFC family members are expressed during gastrulation in *Xenopus* embryos. Our data indicate that the different Nodal-related ligands require different EGF-CFC family members to signal in gastrulating embryos. We propose that this is important for the regulation and modulation of signalling by Nodal-related ligands during gastrulation.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ydbio.2006.01.006.

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