

***FRL-1*, a member of the EGF-CFC family, is essential for neural differentiation in *Xenopus* early development**

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SUMMARY

Recent studies indicate an essential role for the EGF-CFC family in vertebrate development, particularly in the regulation of nodal signaling. Biochemical evidence suggests that EGF-CFC genes can also activate certain cellular responses independently of nodal signaling. Here, we show that *FRL-1*, a *Xenopus* EGF-CFC gene, suppresses BMP signaling to regulate an early step in neural induction. Overexpression of *FRL-1* in animal caps induced the early neural markers *zic3*, *soxD* and *Xngnr-1*, but not the pan-mesodermal marker *Xbra* or the dorsal mesodermal marker *chordin*. Furthermore, overexpression of *FRL-1* suppressed the expression of the BMP-responsive genes, *Xvent-1* and *Xmsx-1*, which are expressed in animal caps and induced by overexpressed *BMP-4*. Conversely, loss of function analysis using morpholino-antisense oligonucleotides against *FRL-1* (FRL-1MO) showed that *FRL-1* is required for neural development. FRL-1MO-injected embryos lacked neural structures but contained mesodermal tissue. It was suggested previously that

expression of early neural genes that mark the start of neuralization is activated in the presumptive neuroectoderm of gastrulae. FRL-1MO also inhibited the expression of these genes in dorsal ectoderm, but did not affect the expression of *chordin*, which acts as a neural inducer from dorsal mesoderm. FRL-1MO also inhibited the expression of neural markers that were induced by *chordin* in animal caps, suggesting that *FRL-1* enables the response to neural inducing signals in ectoderm. Furthermore, we showed that the activation of mitogen-activated protein kinase by FRL-1 is required for neural induction and BMP inhibition. Together, these results suggest that *FRL-1* is essential in the establishment of the neural induction response.

Key words: *Xenopus*, *FRL-1*, EGF-CFC family, *cripto*, *cryptic*, *one-eyed pinhead*, Neural induction, Default model, Bone morphogenetic protein, Fibroblast growth factor, Ras/Raf/MAPK signaling

INTRODUCTION

The EGF-CFC family of proteins are secreted signaling molecules important in early vertebrate development. They have a variant Epidermal Growth Factor (EGF)-like domain and a CFC domain that is conserved among mouse *Cripto*, *Xenopus FRL-1* and mouse *Cryptic* (Shen and Schier, 2000). EGF-CFC proteins contain a hydrophobic C terminus and are connected to the cell membrane via a glycosylphosphatidylinositol (GPI) linkage (Zhang et al., 1998; Minchiotti et al., 2000). The EGF-CFC family of proteins include human *Cripto* and *Cryptic*, mouse *Cripto* and *Cryptic*, chicken *Cripto*, *Xenopus FRL-1* and zebrafish *One-eyed pinhead* (*Oep*) (Ciccociola et al., 1989; Dono et al., 1993; Shen et al., 1997; Colas and Schoenwolf, 2000; Kinoshita et al., 1995; Zhang et al., 1998). Loss-of-function experiments in zebrafish show that the *one-eyed pinhead* (*oep*) mutant phenotype is similar to that of the zebrafish double mutants of

the *nodal*-related genes, *squint* and *cyclops*, indicating that the EGF-CFC gene is an essential cofactor in nodal signaling (Gritsman et al., 1999; Schier and Shen, 2000).

The *Xenopus* EGF-CFC protein FRL-1 was isolated as a ligand of the fibroblast growth factor (FGF) receptor and is considered a member of the EGF-CFC protein family (Kinoshita et al., 1995; Shen et al., 1997). *FRL-1* is expressed ubiquitously during gastrulation and can induce neural and mesodermal markers in presumptive ectoderm (Kinoshita et al., 1995). Studies of FGF signaling suggest the existence of two signal transduction pathways mediated by the FGF receptor. First, activation of the FGF receptor can activate the phospholipase C- γ (PLC γ) pathway to produce inositol triphosphate (InsP₃) and facilitate Ca²⁺ release (reviewed by Powers, 2000). Second, the FGF receptor also signals to Ras, which subsequently activates the mitogen-activated protein kinase (MAPK) signaling pathway (reviewed by Powers, 2000). It remains unclear which of these pathways interacts with FRL-1.

In vertebrate early development, bone morphogenetic protein (BMP) signaling stimulates epidermal induction of undifferentiated cells in the presumptive ectoderm region and inhibits neural induction (Hemmati-Brivanlou and Melton, 1997). Neural cells are induced in the presumptive ectoderm when BMP signaling is inhibited by factors such as Noggin, Chordin and Follistatin, which are secreted from the organizer region that differentiates into axial mesoderm. Initially, neural-inducing signals from the organizer are thought to act in an instructive manner. They can, however, also bind directly to BMP proteins. This leads to the 'default model' of neural induction, which proposes that in the absence of cell-cell signaling, ectodermal cells will adopt a neural fate (Munoz-Sanjuan and Hemmati-Brivanlou, 2002).

In this study, we have examined FRL-1 function in early *Xenopus* development and find that it acts as a neural inducer. FRL-1 inhibits BMP signaling via the activation of MAPK signaling, implicating it early in neural induction. These data indicate that FRL-1-induced neural differentiation may occur via a nodal-independent mechanism.

MATERIALS AND METHODS

Embryos

Eggs were obtained by injecting human chorionic gonadotropin (Gestron: Denka Seiyaku) into *Xenopus laevis*. Fertilized eggs were obtained by artificial insemination and dejellied using 1% thioglycolate in Steinberg's solution. Microinjection was carried out according to the method of Tanegashima et al. (Tanegashima et al., 2000). All injection experiments were performed more than two times independently. In the animal cap assay, mRNAs were injected into each blastomere of 4-cell-stage embryos, whose animal caps were dissected with fine needles on a 0.5 mm square and cultured in 100% Steinberg's solution with 0.1% bovine serum albumin until sampling. For western blot analysis of MAPK, animal caps were dissected in Ca²⁺- and Mg²⁺-free modified Barth's solution to prevent wounding. Embryos were staged according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1956).

Constructs

PBluescript *FRL-1* (pBS-*FRL-1*) was obtained by screening our cDNA library containing maternal genes. The construction of pCS2-*FRL-1Δ5'UTR* was performed by amplifying the *FRL-1* ORF using PCR. For the construction of pCS2-*FRL-1*, which contains the 5'UTR region, *EcoRI*- and *XhoI*-digested pBS-*FRL-1* was ligated into pCS2 vector. To test FRL-1 function (Figs 1, 10 and 11), we used pCS2-*FRL-1Δ5'UTR* as *FRL-1* because of its strong activity. For construction of pCS2-*FRL-1Δ5'UTR*-6myc and pCS2-*FRL-1*-6myc, *FRL-1* cDNA fragments were amplified by PCR from pCS2-*FRL-1Δ5'UTR* and pCS2-*FRL-1*, respectively and the amplified products were ligated into pCS2+6myc vector. pCS2-*FRL-1ΔCFC*-6myc and pCS2-*FRL-1ΔEGF*-6myc constructs coded FRL-1-6myc protein without CFC domain (116Cys-stop codon), and EGF domain (77Lys-112Arg), respectively. For construction of pCS2-*oep*, *oep* ORF was amplified from pCDNA-*oep*-flag (Zhang et al., 1998) and ligated into pCS2+ vector. For construction of pCS2-chickCFC, pKS-chickCFC (Andree et al., 2000) was digested with *PstI*. The released fragments were blunt-ended and ligated into pCS2 vector. For the preparation of mRNA, pCS2-*chordin*, pCS2-*lacZ* (Takahashi et al., 2000), pCS2-*FRL-1*, pCS2-*FRL-1Δ5'UTR*, pCS2-*FRL-1*-6myc, pCS2-*FRL-1Δ5'UTR*-6myc, pCS2-*FRL-1ΔCFC*-6myc, pCS2-*FRL-1ΔEGF*-6myc, pCS2-*cripto* (Yeo and Whitman, 2001), pSP64T-*BMP-4*, pSP64T-*XFD* (Amaya et al., 1991) and pSP64T-*SESE-MAPKK* (Gotoh et al., 1995) were

linearized and transcribed using the mMESSAGE mMACHINE SP6 kit (Ambion).

Morpholino oligonucleotides

The FRL-1 morpholinoantisense oligonucleotides (FRL-1MO), FRL-1MO-second and FRL-1-4misMO were designed as follows; FRL-1MO: 5'-AAACTGCATTGTTTTCTGCAAAGGC-3'; FRL-1MO-second: 5'-ATTGAATGTGTCCTTAGCAAAAACC-3', FRL-1-4misMO: 5'-AAACaGCATaGTTTTgTGCAgAGGC-3', lower case letters indicate changes to the FRL-1MO-first oligo sequence (Gene Tools LLC).

RT-PCR and histology

Total RNA isolation, RT-PCR and histological analyses were performed as described previously (Tanegashima et al., 2000). The primer pairs used were as follows; *soxD* (forward, 5'-TCAGCA-ACAGGTCCAGTACC-3'; reverse, 5'-TCTAACAAGATCCGACGCC-3'), *FRL-1* (forward, 5'-ATGCAGTTTTTAAGATTT-3'; reverse, 5'-TTAAAGTCCAATATT-3'), *endodermin* (*edd*) (forward, 5'-TAT-TCTGACTCCTGAAGGTG-3'; reverse, 5'-GAGAAGTGCCTATG-TGCCTC-3'), *collagen type II* (*col II*) (forward, 5'-ATTCACTG-ACCTTCCTGCG-3'; reverse, 5'-TCCATAGGTGCAATGTCTACG-3'), *Xngnr-1* (forward, 5'-CGCCGCAACCCGACTCACCT-3'; reverse, 5'-CCTGCATCGCGGGCTGTTCTC-3'), *Xvent-1* (forward, 5'-TTCCCTTCAGCATGGTTCAAC-3'; reverse, 5'-GCATCTCT-TGGCATATTTGG-3'). The primer pairs for *chordin*, *Xmsx-1*, β -*crystallin*, *ms-actin*, *EF1- α* , *otx2*, *zic3*, *N-tubulin*, *Xbra* and *N-CAM* were as described in Xenopus Molecular Marker Resource (<http://www.cbrmed.ucalgary.ca/pvize/html/WWW/Welcome.html>). *EF1- α* was used as a loading control. Reverse transcriptase negative (RT-) reactions were performed to indicate the absence of contaminating genomic DNA.

Whole-mount in situ hybridization

Analysis of whole-mount in situ hybridization was carried out as described previously (Harland, 1991) using digoxigenin-labeled antisense probes. For cell-lineage tracing, 20 ng of FRL-1MO or 20 ng of FRL-1-4misMO were co-injected with 250 pg of *lacZ* into one animal blastomere of albino embryos. Injected regions were stained with red gal (Research Organics, Inc, USA). Probes were synthesized using pBS-*chordin* (Sasai et al., 1994), pBS-*zic3*, pBS-*soxD*, pGEM-*otx2*, pBS-*Xngnr-1* (Ma et al., 1996) and pBS-*FRL-1* as templates. pBS-*soxD* and pBS-*zic3* were obtained by our own screening.

Western blotting

FRL-1 proteins with 6myc-epitope tags were detected using the 9E10 monoclonal antibody (Santa Cruz). α -actin served as the loading control and was detected by the AC-40 monoclonal antibody (Sigma). Activated MAPK was detected by monoclonal anti-MAPK, activated (diphosphorylated ERK-1 and 2) clone MAPK-YT (Sigma) and total MAPK protein were detected by ERK2, rabbit polyclonal IgG (Santa Cruz). Anti-mouse IgG, HRP-linked antibody (Cell Signaling Technology, Inc.) and peroxidase-conjugated goat anti-rabbit IgG (Sigma) were used as the secondary antibodies.

Whole-mount in situ immunohistology

Neu-1 monoclonal antibody was described previously (Itoh and Kubota, 1989). After detection, wild-type embryos with Neu-1 staining were bleached. For the detection of activated MAPK, whole-mount in situ immunohistology was done using monoclonal anti-MAPK, activated (diphosphorylated ERK-1 and 2) clone MAPK-YT (SIGMA). The signal was detected using BM purple (Roche).

Proteins and chemical compounds for treatment

Purified bFGF (Amersham Biosciences), PD98059 (Biomolecules for

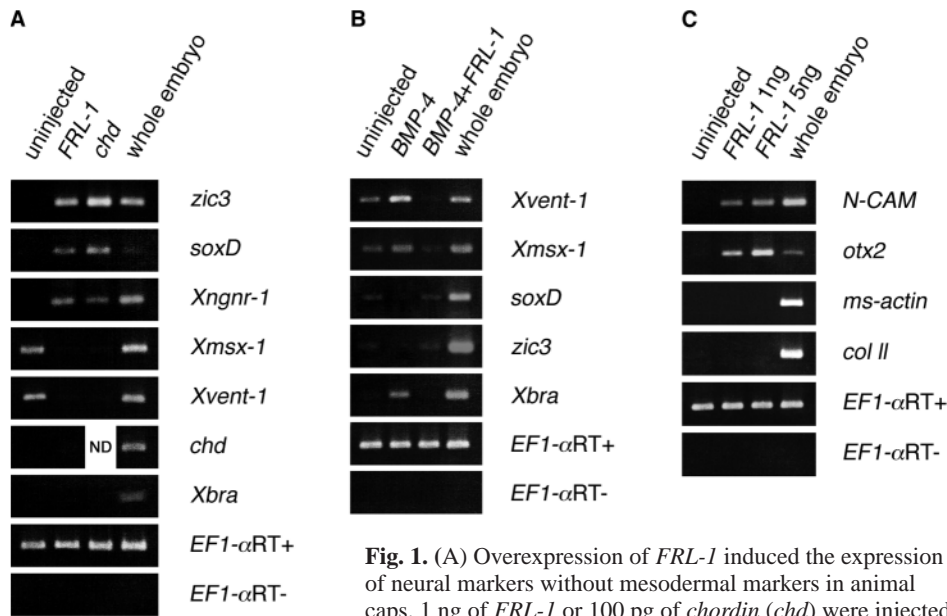


Fig. 1. (A) Overexpression of *FRL-1* induced the expression of neural markers without mesodermal markers in animal caps. 1 ng of *FRL-1* or 100 pg of *chordin* (*chd*) were injected into animal caps, which were dissected at stage 9, together

with an uninjected control, and harvested at stage 13 with the whole embryos for RT-PCR analysis. *FRL-1* induced the expression of *zic3*, *soxD*, *Xngnr-1* without induction of *Xbra* and *chordin*, and inhibited *Xmsx-1* and *Xvent-1*. 'ND' indicated no data. (B) *FRL-1* inhibits BMP signaling in animal caps. Animal caps injected with 1 ng of *BMP-4* with or without 1 ng of *FRL-1*, were dissected at stage 9 and harvested at stage 10.5 for RT-PCR. The induction of *Xvent-1*, *Xmsx-1* and *Xbra* induced by *BMP-4* was inhibited by co-injection of *FRL-1*, and the inhibition of *zic3* and *soxD* by *BMP-4* was rescued by co-injection of *FRL-1*. (C) *FRL-1* induced the expression of neural markers, *N-CAM* and *otx2* in a concentration-dependent manner without induction of mesodermal markers, *muscle specific-actin* (*ms-actin*) and *collagen type II* (*col II*). 1 ng or 5 ng of *FRL-1*-injected animal caps were dissected at stage 9 with the uninjected control and harvested at stage 26 for RT-PCR.

as does the BMP antagonist, *chordin* (Fig. 1A, lane 3), suggesting that *FRL-1* acts as a neural inducer by inhibiting BMP signaling.

To test this hypothesis, we used the animal cap assay to examine whether *FRL-1* inhibited early BMP response genes induced by *BMP-4* (Koster et al., 1991). *BMP-4* induced the expression of *Xmsx-1*, *Xvent-1* and *Xbra* at the early gastrula stage (Fig. 1B, lane 2), whereas *FRL-1* completely inhibited the expression of BMP response genes induced by *BMP-4* (Fig. 1B, lane 3).

Under our experimental conditions, *FRL-1* induced neural induction at an early stage. We also tested whether *FRL-1* induced the expression of late neural markers and mesodermal markers. *FRL-1* induced the expression of *N-CAM* and the anterior neural marker *otx2* (Pannese et al., 1995) in a concentration-dependent manner, but not *ms-actin* and *collagen type II* (Su et al., 1991), which were expressed in muscle and notochord, respectively, even when *FRL-1* was used at high doses (Fig. 1C). These results indicate that *FRL-1* can directly induce anterior neural tissue without the induction of mesoderm.

Research Success) and LY294002 (Biomolecules for Research Success) were purchased commercially. PD98059 and LY294002 were dissolved in DMSO and stored at 10 mM concentration.

RESULTS

FRL-1 causes neural induction by inhibiting BMP signaling

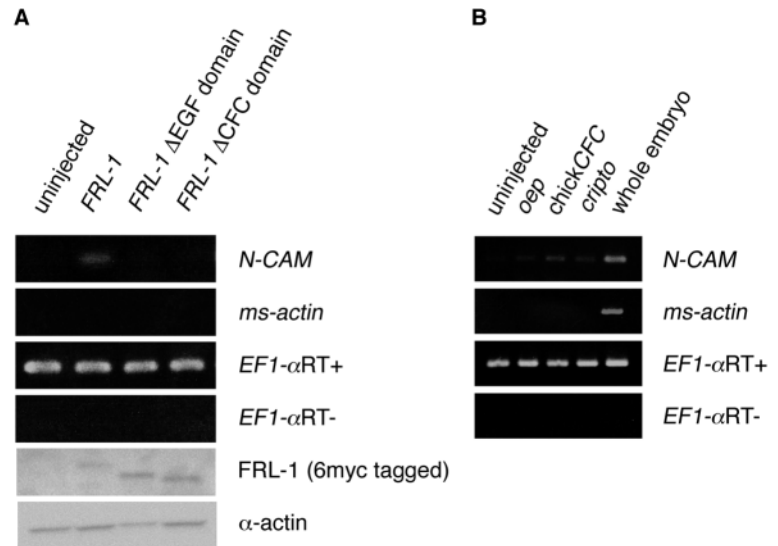
FRL-1 induces expression of the pan-neural marker, *N-CAM* (Kintner and Melton, 1987) in animal caps and the mesodermal marker, *muscle specific-actin* (*ms-actin*) (Stutz and Spohr, 1986) at high doses (Kinoshita et al., 1995). However, it is unclear how *FRL-1* functions in the earlier stages of neural induction. To analyze this, we investigated whether *FRL-1* induces the expression of early neural and mesodermal markers in animal caps. RT-PCR analysis indicated that 1 ng of *FRL-1* induced the early neural markers, *zic3* (Nakata et al., 1997), *soxD* (Mizuseki et al., 1998) and *Xngnr-1* (Ma et al., 1996) but not the pan-mesodermal marker *Xbra* (Smith et al., 1991) or the dorsal mesodermal marker *chordin* (Sasai et al., 1994) in animal caps at the early neurula stage (Fig. 1A, lane 2). In addition, *FRL-1* suppressed the expression of BMP responsive genes, *Xvent-1* (Gawantka et al., 1995) and *Xmsx-1* (Maeda et al., 1997; Suzuki et al., 1997) (Fig. 1A, lane 2). These results indicate that overexpression of *FRL-1* causes neural induction without mesoderm induction

EGF-CFC proteins show conserved function in neural induction

EGF-CFC proteins have two conserved motifs, the EGF-like and the CFC motif (Shen and Schier, 2000). To examine the function of these motifs in neural induction, two cDNA constructs encoding an EGF-like motif-deleted form with the 6myc-epitope tag (*FRL-1ΔEGF* domain) and a CFC motif-deleted form with the 6myc-epitope tag (*FRL-1ΔCFC* domain) were generated and used for the animal cap assay. Western blotting confirmed expression of these protein products in the injected animal caps. Overexpression of mRNA encoding *FRL-1* with 6myc-epitope tag in ectoderm induced the neural marker *N-CAM*. However, the *FRL-1ΔEGF* domain or *FRL-1ΔCFC* domain showed no such inductive ability (Fig. 2A). This suggests that both the EGF-like and CFC domains of *FRL-1* are required for neural induction.

We next examined whether the neural induction activity of *FRL-1* was conserved among the other EGF-CFC genes (Fig. 2B). Overexpression of *oep* (a zebrafish EGF-CFC gene) neuralized animal caps, as previously reported (Kiecker et al., 2000), while overexpression of other EGF-CFC genes, mouse *cripto*, and chick *CFC* (chick *cripto*) induced the expression of neural marker, *N-CAM* without the induction of mesodermal marker, *ms-actin*. The *N-CAM* expression induced by these EGF-CFC genes was weaker than that induced by *FRL-1* (data not shown), which may reflect receptor differences between

Fig. 2. (A) Both the EGF and CFC domain are necessary for FRL-1-mediated induction of expression of neural markers. 1 ng of *FRL-1*-6myc, which encodes for FRL-1 tagged with six c-myc-epitope (*FRL-1*), the EGF domain-deleted form of *FRL-1*-6myc (*FRL-1*ΔEGF domain) or the CFC domain-deleted form of *FRL-1*-6myc (*FRL-1*ΔCFC domain) was injected into each animal blastomere of 4-cell-stage embryos. Animal caps were dissected at stage 9 with an uninjected control and harvested at stage 25 for RT-PCR. Western blotting analysis showed FRL-1-6myc, FRL-1ΔEGF domain-6myc and FRL-1ΔCFC domain-6myc proteins were generated in animal caps at stage 10.5. Although *FRL-1* induced the expression of *N-CAM*, it was not detected in the *FRL-1*ΔEGF domain and *FRL-1*ΔCFC domain mRNA-injected animal caps. (B) Activity of neural induction by *FRL-1* was conserved among other members of the EGF-CFC family in vertebrates. Animal caps injected with 2 ng of zebrafish *oep*, mouse *cripto* or chick *CFC* (chick *cripto*) were dissected at stage 9 with an uninjected control and harvested at stage 25 with whole embryos for RT-PCR. The data showed that not only *FRL-1* but also *oep*, chick *CFC*, and mouse *cripto* induced the expression of *N-CAM* without *ms-actin*.



species. Overall, our results suggest that the function in neural induction is conserved among EGF-CFC genes.

FRL-1MO specifically inhibits translation of *FRL-1*

We clearly show that *FRL-1* acts as a neural inducer by inhibiting BMP signaling in the animal cap. To study the role of *FRL-1* in neural induction in *Xenopus* embryos, we generated an antisense morpholino oligonucleotide against *FRL-1* (*FRL-1*MO) and then tested whether it specifically inhibited the translation of *FRL-1* using the *Xenopus* oocyte expression system (Fig. 3). First, FRL-1-6myc proteins, consisting of FRL-1 tagged with the 6myc-epitope at the C-terminal region were detected at approximately 35 kDa and 25 kDa (Fig. 3, lanes 2, 5). A 25 kDa protein is consistent with the predicted molecular size of the FRL-1-6myc protein. The 35 kDa band may represent a glycosylated form of FRL-1-6myc since the mouse EGF-CFC protein, Cripto is known to be fucosylated (Schiffer et al., 2001). FRL-1MO was shown to inhibit the translation of *FRL-1* (Fig. 3, lane 3), but not FRL-1-4misMO, which contains four nucleotide substitutions to exclude the toxicity of MO or *FRL-1*Δ5'UTR that lacks the target sequence of FRL-1MO (Fig. 3, lanes 4, 6). These results imply that the FRL-1MO specifically inhibited FRL-1 translation.

FRL-1 is required for neural induction in *Xenopus* embryos

To confirm our results with misexpression of *FRL-1*, we performed a loss-of-function study by microinjecting FRL-1MO into *Xenopus* embryos (Fig. 4). Embryos that had been injected anally into each blastomere at the 8-cell stage with 15 ng of FRL-1MO showed head defects with no eye structures (Table 1, Fig. 4B). Injection of 15 ng of FRL-1-4misMO anally or with 15 ng of FRL-1MO vegetally had no apparent inhibitory effects (Table 1). We also generated a second antisense MO against *FRL-1*, which was designed against another region of the 5'UTR of the *FRL-1* mRNA (*FRL-1*MO-second). Embryos injected with 40 ng of FRL-1MO-second

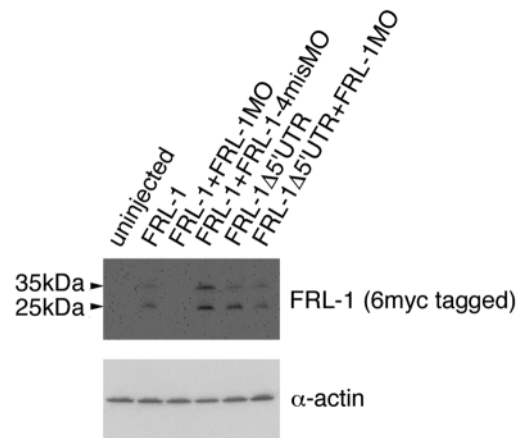


Fig. 3. FRL-1MO specifically inhibited the translation of *FRL-1*. 10 ng of *FRL-1*-6myc mRNA, which codes for FRL-1 tagged with six c-myc-epitopes was injected with or without 40 ng of FRL-1MO or FRL-1-4misMO into oocytes. 10 ng of *FRL-1*Δ5'UTR-6myc lacking the target sequence of FRL-1MO, was also injected with or without 40 ng of FRL-1MO. Lysates with uninjected controls were used for western blotting. FRL-1 proteins with the 6myc-epitope tag were detected by anti-c-myc antibody. The translation of *FRL-1* was inhibited by FRL-1MO but not by FRL-1-4misMO, whereas that of *FRL-1*Δ5'UTR was not inhibited by FRL-1MO.

showed a phenotype similar to those injected with FRL-1MO (Table 1, Fig. 4G). Head structure defects with lack of eyes, caused by microinjection of either FRL-1MO or FRL-1MO-second, could be rescued by co-injection of 20 pg of *FRL-1*Δ5'UTR (Table 1, Fig. 4D,H). These results suggest that the phenotypes caused by microinjection of the MOs against *FRL-1* were due specifically to the depletion of *FRL-1*. Next, we tested the possibility that other EGF-CFC genes or BMP antagonists, such as *chordin*, *noggin* or *FRL-1*ΔEGF and ΔCFC domain could rescue the *FRL-1*-depleted phenotype. The

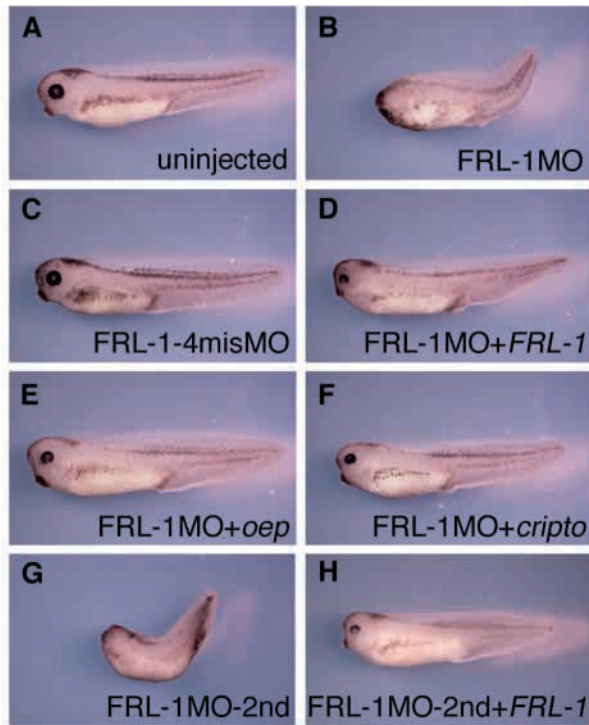


Fig. 4. FRL-1MOs specifically induced head defect phenotypes. FRL-1MOs were injected into animal blastomeres of 8-cell-stage embryos, which were grown to stage 40. (A) Uninjected embryo. (B) Embryo injected with 15 ng FRL-1MO showing defects of the head including lack of eyes. (C) Embryo injected with 15 ng of FRL-1-4misMO showing no defect. (D) Head structure defect induced by 15 ng of FRL-1MO was rescued by co-injection of 20 pg of *FRL-1Δ5'UTR*. (E,F) Some other EGF-CFC genes can rescue the head defect phenotype caused by FRL-1MO. (E) Co-injection with 20 pg of *oep* rescued head defects caused by microinjection of 15 ng of FRL-1MO. (F) Co-injection with 20 pg of mouse *cripto* also rescued head defects. (G,H) FRL-1MO-second suppressed neural formation as well as FRL-1MO. (G) Embryo injected with 40 ng of FRL-1MO-second showing the same phenotype as the FRL-1MO-injected embryo. (H) Head structure defects caused by microinjection of 40 ng of FRL-1MO-second was rescued by co-injection with 20 pg of *FRL-1Δ5'UTR*.

phenotypes of the embryos injected with FRL-1MO were rescued by co-injection with *oep* mRNA (Table 1, Fig. 4E) and *cripto* (Table 1, Fig. 4F), but not with *chordin*, *noggin*, *FRL-1ΔEGF* domain or *FRL-1ΔCFC* domain (Table 1), suggesting that other EGF-CFC genes could compensate for the depletion of *FRL-1*.

Histological analysis was performed to investigate these phenotypes further. The embryos injected with FRL-1MO had neural tissue defects without the loss of mesodermal tissue, such as notochord and pronephric tube (Fig. 5G). However, FRL-1-4misMO-injected embryos showed no significant defects (Fig. 5H). Whole-mount in situ hybridization experiments showed that the expression of *otx2* was completely suppressed by FRL-1MO-injected embryos, confirming that FRL-1MO blocked the formation of anterior neural tissue in *Xenopus* embryo (Fig. 5A). The inhibition of neural induction was also confirmed by whole-mount in situ immunostaining using Neu1 antibody that specifically recognizes neural tissue.

Table 1. FRL-1MOs specifically induced head defect phenotypes

	Numbers	Phenotype (%)	
		Head defect (without eyes)	Normal head
FRL-1-4-misMO 15 ng	50	0	100
FRL-1MO 15 ng	50	90	10
FRL-1MO 15 ng (veg)	50	0	100
FRL-1MO 15 ng	50	18	82
+ <i>FRL-1Δ5'UTR</i> 20 pg			
FRL-1MO 15 ng	50	96	4
+ <i>FRL-1ΔEGF</i> 20 pg			
FRL-1MO 15 ng	51	98	2
+ <i>FRL-1ΔCFC</i> 20 pg			
FRL-1MO 15 ng	52	31	69
+ <i>oep</i> 20 pg			
FRL-1MO 15 ng	52	29	71
+ <i>cripto</i> 20 pg			
FRL-1MO 15 ng	50	94	6
+ <i>chordin</i> 20 pg			
FRL-1MO 15ng	50	88	12
+ <i>noggin</i> 20 pg			
FRL-1MO 15 ng	52	21	79
+ <i>CA-MAPKK</i> 2 ng			
FRL-1-4-misMO 40 ng	50	2	98
FRL-1MO-second 40 ng	50	88	12
FRL-1MO-second 40 ng	51	22	78
+ <i>FRL-1Δ5'UTR</i> 20 pg			

Note: MOs and mRNAs were animaly injected into each blastomere of 8-cell-stage embryos, except where denoted by (veg) which indicates vegetal injection.

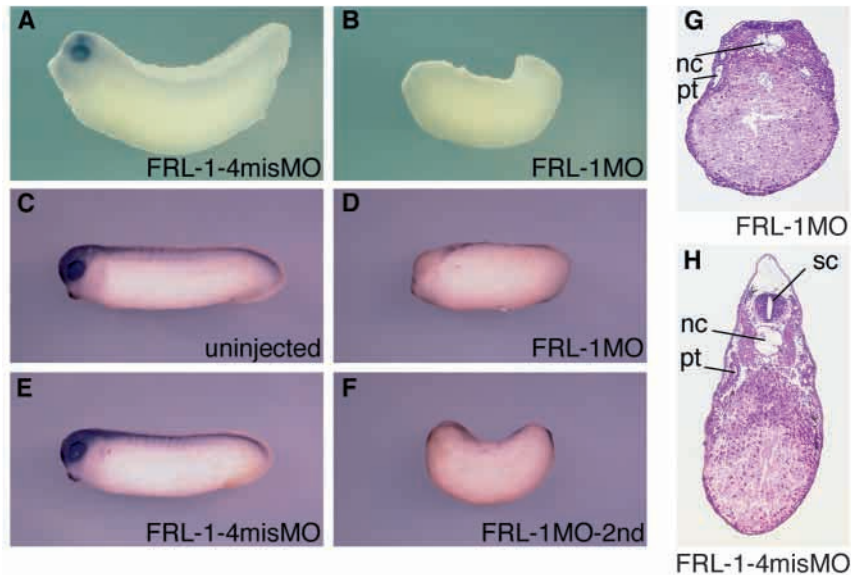
The staining showed that the injection of both FRL-1MO and FRL-1MO-second inhibits neural induction but not FRL-1-4misMO (Fig. 5D-F). RT-PCR analysis also showed that *FRL-1Δ5'UTR* could rescue the FRL-1MO-induced suppression of neural markers, *N-CAM*, *otx2*, *β-crystallin* (Altmann et al., 1997) and *N-tubulin* (Richter et al., 1988; Good et al., 1989; Hartenstein, 1989), but not the mesodermal markers, *ms-actin* and *collagen type II* and endodermal marker, *endodermin* (Sasai et al., 1996) (Fig. 6).

To investigate whether *FRL-1* is involved in neural induction at an early stage, we observed the expression of early neural genes in *FRL-1*-depleted embryos (Fig. 7). FRL-1MO (20 ng) or FRL-1-4misMO (20 ng) was co-injected with cell lineage tracer, *lacZ* into one blastomere of 4-8 cell-stage embryos, which were cultured until early gastrula stage for *chordin* and early neurula stage for *zic3*, *soxD* and *Xngnr-1*. Whole-mount in situ hybridization analysis showed that the expression of early neural markers, *zic3*, *soxD* and *Xngnr-1* was suppressed in the FRL-1MO-injected regions that were stained red for β-galactosidase activity (Fig. 7C-H), suggesting that FRL-1MO inhibits the early step of neural induction. However, the expression of *chordin*, which acts as a neural inducing signal from dorsal mesoderm, was not suppressed in the FRL-1MO-injected region (Fig. 7A,B). Therefore, these results indicate that *FRL-1* is required for neural differentiation at an early stage, and does not affect the expression of the neural inducer, *chordin*.

***FRL-1* confers competence to respond to neural inducing signals in the ectoderm**

Neural induction occurs autonomously when BMP signaling is

Fig. 5. Neural induction is suppressed in FRL-1MO-injected embryos. (A,B) FRL-1MO or FRL-1-4misMO was injected into animal blastomeres of 8-cell-stage embryos, which were fixed at stage 30. (A) Embryo injected with 10 ng of FRL-1-4misMO. Transcripts of *otx2* were detected in the pineal gland and in the eye. (B) Embryo injected with 10 ng of FRL-1MO, showing no expression of *otx2*, confirming the loss of anterior neural tissue. (C-F) FRL-1MO, FRL-1-4mis-MO or FRL-1MO-second was injected into animal blastomeres of 8-cell-stage embryos, which were fixed at stage 30 for whole-mount in situ immunohistochemistry analysis using Neu-1. (C) Uninjected embryo. (D) Embryo injected with 15 ng of FRL-1MO. (E) Embryo injected with 40 ng of FRL-1-4misMO. (F) Embryo injected with 40 ng of FRL-1MO-second. (G,H) Histological analysis shows that the embryos injected with FRL-1MO had neural tissue defects without loss of mesodermal tissue, such as notochord and pronephric tube. nc; notochord, pt; pronephric tube, sc; spinal cord. FRL-1MO or FRL-1-4misMO was injected into animal blastomeres of 8-cell-stage embryos, which were fixed at stage 40. (G) Histological section of 10 ng of FRL-1MO-injected embryos. (H) Histological section of embryo injected with 10 ng of FRL-1-4misMO.



inhibited in the presumptive ectoderm of *Xenopus* embryos (Munoz-Sanjuan and Hemmati-Brivanlou, 2002), suggesting that ectodermal cells originally are competent to adopt a neural fate and that a set of genes to drive that fate is stored in the presumptive ectoderm maternally. *FRL-1* is expressed maternally and ubiquitously in *Xenopus* early gastrulae (Kinoshita et al., 1995) and is required for neural induction during the early stages of embryogenesis. Therefore, it was

thought that *FRL-1* might confer the neural fate competence in the ectoderm. To test this hypothesis, we examined the response to neural inducing signals in *FRL-1*-depleted ectoderm. *Chordin* induced the expression of the neural markers, *N-CAM*, *otx2* and *N-tubulin* in animal caps and the induction was not inhibited by FRL-1-4misMO (Fig. 8, lanes 2, 3). However, FRL-1MO did inhibit the expression of *N-CAM*, *Otx2* and *N-tubulin* in response to the neural inducing signal of *chordin* (Fig. 8, lane 4). The attenuation of neural competence was rescued by *FRL-1Δ5'UTR* (Fig. 8, lane 5). These results indicate that *FRL-1* confers a competence in the ectoderm to respond to neural inducing signals.

***FRL-1* is expressed in neuroectoderm of late gastrula**

FRL-1 is expressed ubiquitously in early gastrula (Kinoshita et al., 1995). However, no spatial expression of *FRL-1* in late gastrula has been reported. We examined the relationship between *FRL-1* expression and neural induction using whole-mount in situ hybridization. Our analysis showed that *FRL-1* was ubiquitously expressed in the early gastrula (Fig. 9A), as previously reported (Kinoshita et al., 1995), whereas it was confined to the presumptive neuroectoderm in late gastrula (Fig. 9B). RT-PCR confirmed the restricted expression of *FRL-1* to presumptive neuroectoderm that was

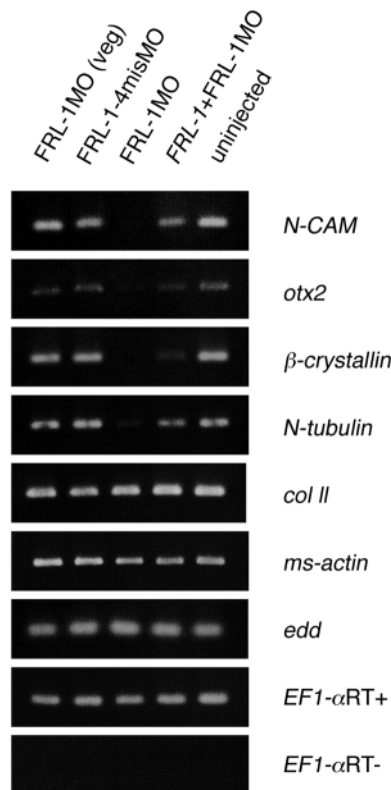


Fig. 6. FRL-1MO inhibits the expression of neural markers without inhibition of mesodermal and endodermal markers in *Xenopus* embryos. 10 ng of FRL-1MO injected into each animal or vegetal blastomere of 8-cell-stage embryos or FRL-1-4misMO injected into each animal blastomere of 8-cell-stage embryos, which were cultured until stage 31 for RT-PCR. FRL-1MO vegetally injected and FRL-1-4misMO animally injected did not affect the expression of any marker when compared with controls (uninjected), whereas FRL-1MO animally injected suppressed the expression of neural markers *N-CAM*, *otx2*, *β-crystallin* and *N-tubulin*. The suppression of neural markers was rescued by 20 pg of *FRL-1Δ5'UTR*.

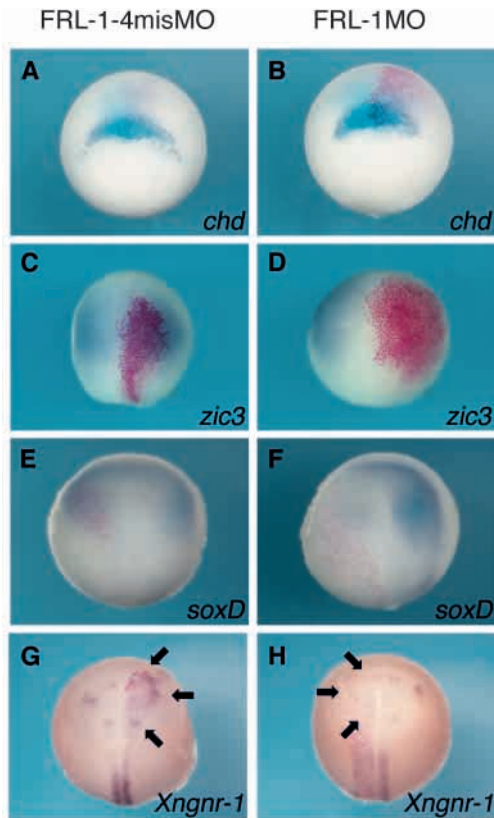


Fig. 7. FRL-1MO inhibits an early step in the neural induction of *Xenopus* embryos. Albino embryos were injected with 20 ng of FRL-1MO (B,D,F,H), or FRL-1-4misMO (A,C,E,G) and 250 pg of *lacZ* into the dorsal marginal zone of a blastomere at the 4-cell-stage for analysis of *chordin* and a dorso-animal blastomere of 8-cell-stage embryos for analysis of *zic3*, *soxD* and *Xngnr-1*. They were cultured until stage 10.5 for whole-mount in situ analysis of *chordin* (A,B) and until stage 13 for analysis of *zic3* (C,D), *soxD* (E,F) and *Xngnr-1* (G,H) and then stained in the MO-injected region with red gal. The suppression of the neural markers *zic3* (D, 77%, $n=22$), *soxD* (F, 84%, $n=37$) and *Xngnr-1* (H, 86%, $n=21$) was observed in the FRL-1MO-injected region whereas *chordin* was not suppressed (B, 0%, $n=29$). However, there was no change in the FRL-1-4misMO-injected region (A, 100%, $n=22$; C, 96%, $n=26$; E, 93%, $n=75$; G, 91%, $n=48$). Arrows in G and H indicate trigeminal ganglia, where the expression of *Xngnr-1* was suppressed in H.

induced by *chordin*. Expression levels of *FRL-1* were higher in *chordin*-injected ectoderm (Fig. 9C, lane 2), which differentiates into neural tissue without mesoderm (Fig. 1A, lane 3; Fig. 9C, lane 2), than in the uninjected ectoderm, when sibling embryos grew to late gastrula. It was previously reported that transcripts of *FRL-1* were expressed until the gastrula stage and that these were rapidly reduced by the end of gastrulation (Kinoshita et al., 1995). Our results suggest that the expression of *FRL-1* is attenuated in presumptive epidermis ahead of the neuroectoderm and is needed to ensure a neural cell fate.

Inhibition of BMP signaling by FRL-1 is required for MAPK activation-associated neural induction

We showed here that *FRL-1* regulates the early step of neural induction by inhibiting BMP signaling (Fig. 1A,B). *FRL-1* in

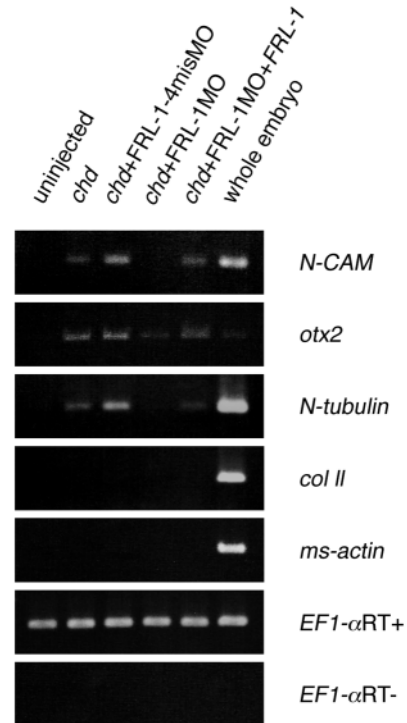


Fig. 8. FRL-1MO inhibits the induction of neural markers by *chordin* in animal caps. 100 pg of *chordin* was injected into the animal pole of 4-cell-stage embryos with 40 ng of FRL-1-4misMO (lane 3), FRL-1MO (lane 4), FRL-1MO and 500 pg of *FRL-1* (lane 5), or without MO (lane 2). The animal caps were dissected at stage 9 and harvested at stage 22 for RT-PCR. *chordin* induces the expression of *N-CAM*, *otx2* and *N-tubulin* without the induction of mesodermal markers *ms-actin* and *collagen type II*, which was inhibited by FRL-1MO but not by FRL-1-4misMO. The suppression of neural markers caused by FRL-1MO was rescued by the co-injection of *FRL-1Δ5'UTR*.

animal caps can be blocked by a dominant-negative FGF receptor-1 construct (*XFD*) (Kinoshita et al., 1995). Our results also confirm that the *N-CAM*-inducing activity of *FRL-1* is required for the FGF receptor signaling pathway (Fig. 10A). Since FGF receptor signaling can activate the MAPK pathway (Powers et al., 2000), we therefore examined whether FRL-1 could activate the MAPK pathway using a monoclonal antibody against activated MAPK. Western blot analysis showed that MAPK activation was upregulated in *FRL-1*-injected animal caps compared with uninjected controls (Fig. 10B). In addition, MAPK activation was detected in the animal caps treated with bFGF, a known activator of MAPK signaling, to a similar extent to those injected with *FRL-1* (Fig. 10B). It is known that MAPK is activated in response to wounding (such as dissection) of animal caps. To exclude the possibility that the result was a response to wounding, we tested for MAPK activation by whole-mount immunostaining. *lacZ* with or without *FRL-1* (1 ng) was injected into one blastomere of 2 cell-stage embryos, which were cultured until late blastula stage for whole-mount immunostaining using anti-activated MAPK. Staining for activated MAPK was seen in the region with *FRL-1* (Fig. 10D) but not in the regions with *lacZ* alone (Fig. 10E). We also examined, by western blot analysis, whether MAPK activity was inhibited in the FRL-1-depleted

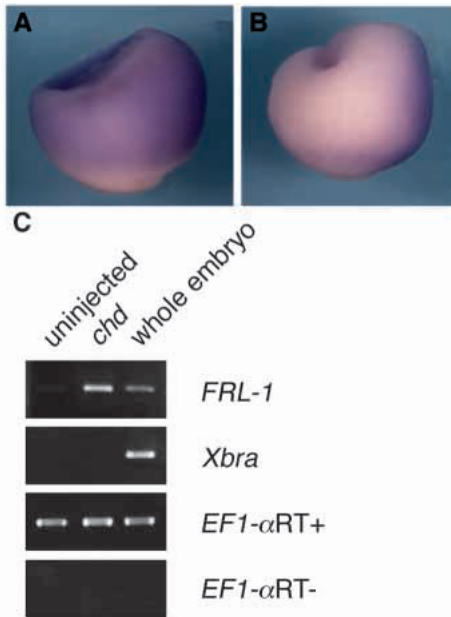
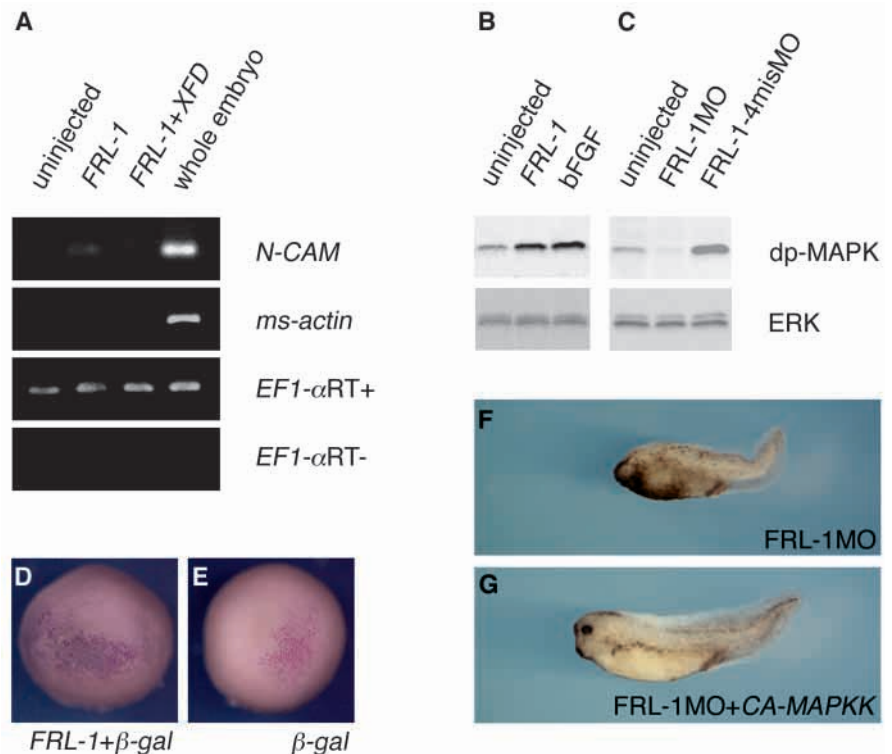


Fig. 9. *FRL-1* expression in neuroectoderm at the late gastrula stage. (A,B) Whole-mount in situ hybridization revealed the spatial expression patterns of *FRL-1*. (A) Lateral view of a stage 10 embryo; right side is dorsal. *FRL-1* was ubiquitously expressed in the early gastrula. (B) Lateral view of a stage 12 embryo; right side is dorsal. Transcripts of *FRL-1* are concentrated in the neuroectoderm. (C) RT-PCR analysis of *chordin*-injected ectoderm. Misexpression of *chordin* induced the expression of *FRL-1* in the absence of the mesodermal marker, *Xbra*.

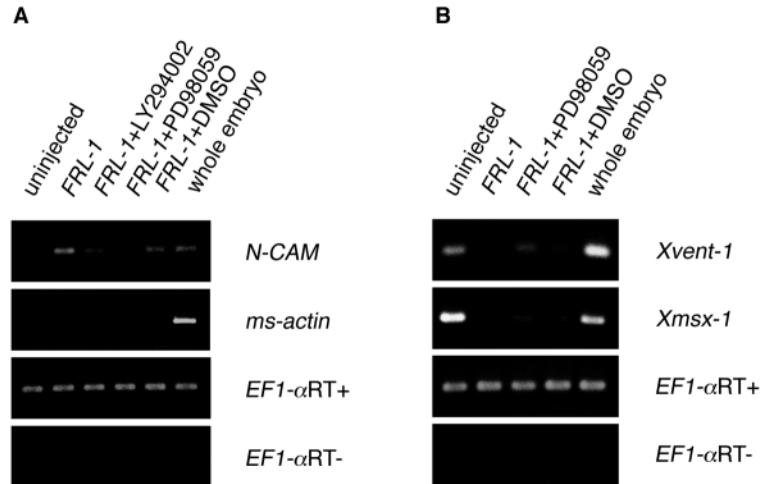
Fig. 10. (A) *FRL-1* caused neural induction via FGF signaling in animal caps. The animal caps that were injected with 1 ng of *FRL-1* alone or co-injected with 2 ng of *XFD*, were dissected at stage 9, together with an uninjected control and harvested at stage 25 with the whole embryos for RT-PCR analysis. *FRL-1* induced the expression of *N-CAM* without induction of *ms-actin*, which was inhibited by *XFD*. (B) *FRL-1* stimulated the MAPK activity. 1 ng of *FRL-1* was injected into each animal blastomere of 4-cell-stage embryos, which were dissected at stage 9 with an uninjected control in Ca^{2+} - and Mg^{2+} -free MBS and harvested at stage 10.5 for western blotting. bFGF treatment (100 ng/ml) served as a positive control. *FRL-1* induced MAPK activation to the same extent as bFGF, and to a greater extent than in uninjected caps. Total MAPK protein (ERK) was detected and used as a loading control. (C) Injection of *FRL-1*-MO downregulated MAPK activity. Animal caps injected with 20 ng of *FRL-1*-MO or *FRL-1*-4misMO were dissected at stage 9 with the uninjected control and harvested at stage 10.5 for western blotting. (D,E) MAPK activity was enhanced in *FRL-1*-injected embryos. Activation of MAPK was detected by whole-mount immunohistology. 500 pg of *lacZ* mRNA was injected with (D) and without (E) 1 ng of *FRL-1* into one blastomere of 2-cell-stage embryos. Injected embryos were grown to stage 9. The injected regions were visualized by red gal staining and the MAPK activated-region was visualised by blue staining. (F,G) Activated MAPK rescued the phenotype caused by *FRL-1*-MO. Head structure defects induced by 15 ng of *FRL-1*-MO were rescued by co-injection of 2 ng of *SESE-MAPKK* (constitutively-activated MAPKK).



region. In 2 of 3 experiments, MAPK activation was strongly downregulated in *FRL-1*-MO-injected animal caps (Fig. 10C), but was unaffected in the third experiment (data not shown). This result suggested that the phenotype seen with *FRL-1*-MO injection may be caused by downregulation of MAPK activation. To confirm this, we attempted to rescue this phenotype by expression of the constitutively activated form of MAPKK, an intracellular activator of MAPK. Indeed, this did rescue the *FRL-1*-MO phenotype, suggesting that *FRL-1* regulates ectodermal MAPK activity to induce neural induction (Fig. 10F,G).

To further examine whether MAPK activation is required for *FRL-1* regulation of neural induction, we used a MEK inhibitor, PD98059, to inhibit MAPK activation by MEK. In animal caps, the expression of *N-CAM* induced by the overexpression of *FRL-1* was inhibited by treatment with PD98059, suggesting that *FRL-1* is required for MAPK activation to induce the expression of neural markers (Fig. 11A). In contrast, a specific inhibitor of PI3K, LY294002 did not affect the *N-CAM* induction by *FRL-1* (Fig. 11A), even though the FGF receptor is known to activate PI3K signaling (Powers et al., 2000). We also showed that *FRL-1* acts as a neural inducer by inhibiting BMP signaling. We therefore examined whether inhibition of BMP signaling by *FRL-1* was affected by the MEK inhibitor, PD98059 (Fig. 11B). RT-PCR analysis showed that the expression of BMP responsive genes, *Xmsx-1* and *Xvent-1*, which was suppressed by *FRL-1* in animal caps, were rescued by treatment with PD98059 (Fig. 11B). These data suggest that inhibition of BMP signaling by *FRL-1* is required for MAPK activation.

Fig. 11. (A) FRL-1 enhances MAPK activity to cause neural induction in animal caps. Animal caps injected with 1 ng of *FRL-1* were dissected at stage 8, together with an uninjected control (lane 1). *FRL-1*-injected animal caps were cultured in 100% Steinberg's solution (SS) with 0.1% bovine serum only (0.1% BSA in SS) (lane 2), or with LY294002 (20 μ M), a specific inhibitor of phosphatidylinositol 3 kinase, in 0.1% BSA in SS (lane 3) or with PD98059 (20 μ M), a inhibitor of MEK (MAPKK), in 0.1% BSA in SS (lane 4) or with dimethyl sulfoxide (DMSO) in 0.1% BSA in SS (lane 5) until sampling, then harvested at stage 25 with the whole embryos for RT-PCR. Since PD98059 and LY294002 were dissolved in DMSO, DMSO treatment is a control to exclude DMSO effects. *FRL-1* induced the expression of *N-CAM* (lane 2), and this was inhibited by treatment with PD98059 but not LY294002, compared with DMSO alone. (B) FRL-1 inhibition of BMP signaling is required for MAPK activation. *FRL-1* or uninjected animal caps were dissected at stage 8 and treated with or without PD98059 or DMSO only. The animal caps and control embryos were harvested at stage 12. The expression of BMP responsive genes, *Xvent-1* and *Xmsx-1* were inhibited by injection of *FRL-1* whereas the inhibition of *Xvent-1* and *Xmsx-1* was rescued by treatment with PD98059.



DISCUSSION

Members of the EGF-CFC protein family play an essential role in vertebrate development. In particular, they are required for nodal signaling, which controls mesendoderm induction, anterior-posterior patterning and left-right asymmetry (Schier and Shen, 2000). Biochemical studies have revealed that EGF-CFC proteins are involved not only in nodal signaling but also in another signaling pathway (Shen and Schier, 2000). In this study, we show that overexpression of *FRL-1* induces early neural genes by inhibiting BMP signaling, and that loss of *FRL-1* function causes neural defects during development. These results suggest that *FRL-1* is essential in neural development.

FRL-1 suppresses BMP signaling to regulate an early step of neural induction

The expression of early neural genes such as *soxD*, *sox2*, and *zic*-related genes is activated in the presumptive neuroectoderm of gastrulae, and signals the start of neuralization (Sasai, 2001). In animal cap assays, we now show that overexpression of *FRL-1* induces *soxD* and *zic3*, and *FRL-1*IMO inhibits the induction of these genes in neuroectoderm, suggesting that *FRL-1* regulates an early step of neural induction. Our studies also suggest that *FRL-1*IMO-injected ectoderm loses competence to respond to the neural inducer, *chordin*, which acts as an antagonist of BMP. In *Xenopus*, the 'default model' proposes that neural induction occurs only by BMP inhibition in the presumptive ectoderm (Munoz-Sanjuan and Hemmati-Briavanlou, 2002). However, in chick, misexpression of *chordin* does not induce the expression of neural markers in non-neural ectoderm (Streit et al., 1998), but does induce Hensen's node, which is equivalent to the *Xenopus* organizer (Streit et al., 1998; Streit et al., 2000). These observations indicate that the inhibition of BMP signaling is not sufficient for neural induction and extracellular factors are required in Hensen's node in chick. FGFs are good candidates since FGFs induce neural tissue (Storey et al., 1998; Wilson et al., 2000) and the FGF receptor inhibitor SU5402 inhibits early neural induction in chick (Streit et al., 2000). In *Xenopus*, FGFs also induce

neural tissue (Kengaku and Okamoto, 1993; Lamb and Harland, 1995; Launay et al., 1996) and dominant-negative mutants of FGF receptor type 4a efficiently inhibit anterior neural tissue (Hongo et al., 1999). Although FGF signaling is therefore required in both *Xenopus* and chick, FGFs induce only posterior neural tissue, suggesting that other ligands are responsible for inducing anterior neural tissue. We propose that EGF-CFC proteins are strong candidate neural inducers that can activate FGF signaling. We showed that *FRL-1* induces the expression of anterior neural markers and that *FRL-1*IMO inhibits the induction of anterior neural tissue. In addition, *FRL-1* activates the FGF receptor/MAPK signaling pathway. We note that the expression of chick *CFC* is condensed in Hensen's node region (Colas and Schoenwolf, 2000), in contrast to the ubiquitous expression of *Xenopus FRL-1* (Kinoshita et al., 1995). The expression patterns of chick *CFC* may correspond to a neural inducer in Hensen's node and may reflect the different competence in responding to neural inducers between *Xenopus* and chick.

FRL-1 inhibits BMP signaling via the activation of FGFR/MAPK signaling

Although it has been shown that *FRL-1* activates FGFR-dependent Ca^{2+} release in oocytes and that XFD inhibits *FRL-1* function in animal caps, we show here that *FRL-1* activates MAPK in blastula embryos and its activation is required for BMP inhibition and neural induction by *FRL-1*. Furthermore, we showed both the EGF and CFC domains are necessary for neural induction of *FRL-1* (Fig. 2A). However, the EGF-like domain alone of the EGF-CFC protein Cripto is sufficient to activate the MAPK pathway in mouse mammary epithelial cells (Kannan et al., 1997). This difference may be explained by these proteins binding different receptors: *FRL-1* function may depend on FGF receptor signaling (Kinoshita et al., 1995) (Fig. 10A), whereas in mouse mammary epithelial cells, human Cripto indirectly activates ErbB4 (Kannan et al., 1997; Bianco et al., 1999). These results suggest that both the EGF and CFC domains may be required for FGF receptor-mediated functions of EGF-CFC proteins.

While it is known that EGF-CFC genes are involved in

nodal signaling, in this study, overexpression of *FRL-1* inhibited BMP signaling in animal caps, where *nodal*-related genes are not expressed, suggesting that BMP signaling was blocked in the absence of nodal signaling. We also found that *oep*, mouse *cripto* and chick *CFC* induced the expression of neural markers in *Xenopus* animal caps. Our observations suggest that BMP signaling may not only be inhibited by *FRL-1* but also by EGF-CFC genes in a nodal-independent fashion. Although EGF-CFC proteins are required for nodal signaling, the mouse EGF-CFC protein Cripto activates MAPK signaling, independent of nodal and its receptor, ALK-4 (Bianco et al., 2002). These results suggest that EGF-CFC protein is able to act via MAPK activation, which is independent of nodal. Previous studies showed that activated *Ras* could not rescue *oep* mutant in zebrafish (Gritsman et al., 1999), suggesting that *Oep* function is not required for MAPK activation. *oep* mutants have defects in mesendodermal tissue and are therefore thought to lack a cofactor for nodal. Our results showed that *FRL-1* acts via a MAPK pathway in neural induction (Fig. 11A). These results suggest that a functional EGF-CFC protein is required for MAPK activation in neural induction but not in nodal signaling.

It is known that some extracellular ligands that activate MAPK signaling have opposite effects to BMP. For example, FGF opposes the anti-proliferative effect of BMP-2 during limb bud outgrowth (Niswander and Martin, 1993) and epidermal growth factor (EGF) antagonizes the induction of osteogenic differentiation markers by BMP-2 (Bernier and Goltzman, 1992). Previous reports have suggested that MAPK-mediated phosphorylation negatively regulates the function of Smad1, an intracellular mediator of BMP signaling (Kretzschmar et al., 1997). MAPK phosphorylates the linker domain of Smad1, thereby inhibiting its nuclear translocation and subsequent BMP signaling. This result seems to be applicable for *FRL-1* function in inhibiting BMP signaling and sustaining neural competency. Furthermore, Sater and colleagues showed that MAPK activation is required for the induction of *Xenopus* neuroectoderm (Uzgare et al., 1998; Goswami et al., 2001). Their results suggest that upregulation of MAPK activity is detected in the neuroectoderm of *dominant negative BMPR*- or *noggin*-injected ectoderm and that the overexpression of MAPK phosphatase, which inactivates activated MAPK, inhibits neural induction in *Xenopus* (Goswami et al., 2001). Our results could account for upregulation of MAPK activity by the observed enrichment of *FRL-1* transcripts in neuroectoderm and the induction by *chordin*. Conversely, it was proposed that BMP signaling might inhibit MAPK activity via the TAK1 pathway (Goswami et al., 2001), indicating mutual antagonisms. Taken together, our results suggest that antagonistic effects between the *FRL-1*/FGFR/MAPK pathway and BMP signaling are involved in the establishment of neural versus epidermal cell fate.

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