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# The *Xenopus* receptor tyrosine kinase Xror2 modulates morphogenetic movements of the axial mesoderm and neuroectoderm via Wnt signaling

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## SUMMARY

**The Spemann organizer plays a central role in neural induction, patterning of the neuroectoderm and mesoderm, and morphogenetic movements during early embryogenesis. By seeking genes whose expression is activated by the organizer-specific LIM homeobox gene *Xlim-1* in *Xenopus* animal caps, we isolated the receptor tyrosine kinase *Xror2*. *Xror2* is expressed initially in the dorsal marginal zone, then in the notochord and the neuroectoderm posterior to the midbrain-hindbrain boundary. mRNA injection experiments revealed that overexpression of Xror2 inhibits convergent extension of the dorsal mesoderm and neuroectoderm in whole embryos, as well as the elongation of animal caps treated with activin, whereas it does not appear to affect cell differentiation of neural tissue and notochord. Interestingly, mutant constructs in which the kinase domain was point-mutated or deleted (named Xror2-TM) also inhibited convergent extension, and did not counteract the wild-type, suggesting that the ectodomain of Xror2 per se has activities that may be modulated by the intracellular domain. In relation to Wnt signaling for planar cell polarity, we observed: (1) the Frizzled-like domain in the ectodomain is required for the activity of wild-type Xror2 and Xror2-TM; (2) co-expression of Xror2 with Xwnt11, Xfz7, or both, synergistically inhibits convergent extension in embryos; (3) inhibition of elongation by Xror2 in activin-treated animal caps is reversed by co-expression of a dominant negative form of Cdc42 that has been suggested to mediate the planar cell polarity pathway of Wnt; and (4) the ectodomain of Xror2 interacts with Xwnts in co-immunoprecipitation experiments. These results suggest that Xror2 cooperates with Wnts to regulate convergent extension of the axial mesoderm and neuroectoderm by modulating the planar cell polarity pathway of Wnt.**

Key words: *Xenopus laevis*, Spemann organizer, Convergent extension, Neural plate closure, Planar cell polarity, Xlim-1, Receptor tyrosine kinase, Xror2, Xwnt11, Xfz7, Cdc42

## INTRODUCTION

From the classical transplantation experiments performed in amphibian embryos, the concept of the organizer was developed as the main signaling center that elaborates the vertebrate body plan (Spemann and Mangold, 1924). The Spemann organizer is situated above the dorsal blastopore lip at the beginning of gastrulation, and is fated to become the prechordal plate and notochord as gastrulation proceeds. Two major features of the organizer are the capability of induction (dorsalization of the mesoderm, neuralization of the ectoderm and patterning of the neuroectoderm), and the morphogenetic movements that are described as convergent extension. As a result of the co-operative work of the induction and the morphogenetic movements, the organizer correctly establishes the body plan (Harland and Gerhart, 1997; Keller et al., 1992; Smith and Schoenwolf, 1998). Previous efforts to isolate organizer-specific genes have identified various transcription factors and secreted molecules that are involved in the inducing activities of the organizer (Bouwmeester et al., 1996; Harland, 2000; Harland and Gerhart, 1997; Moon et al., 1997; Sasai et al., 1994; Thomsen, 1997). However, the interactions among these molecules that are required to exert the functions of the organizer have not been fully analyzed, and the molecular study of the morphogenetic movements of the organizer has only recently begun.

Gastrulation in *Xenopus* involves a complex set of morphogenetic movements. The main engine producing the driving force for gastrulation is thought to be convergent extension that results from mediolateral intercalation of the dorsal marginal zone (DMZ), including the Spemann organizer region. While the cellular basis of convergent extension is well documented, molecular mechanisms regulating this process remain poorly understood. It was reported that Wnt5a and Wnt4 affect morphogenetic movements of ectodermal and mesodermal tissues in whole embryos, and inhibit elongation of animal caps treated with a mesodermalizing factor, activin (Moon et al., 1993; Ungar et al., 1995). Wnt11, in *Xenopus* and

zebrafish, has been shown to be required for convergent extension during gastrulation, and the regulation of convergent extension by Wnt11 has been suggested to take place through a non-canonical pathway similar to that involved in planar cell polarity (PCP) signaling in *Drosophila* (Heisenberg et al., 2000; Tada and Smith, 2000). Components of Wnt signaling for the PCP pathway include Frizzled 7 (Xfz7), Strabismus (Stbm), Dishevelled, a Formin Homology Protein called Daam1, and the Rho family GTPases, Rho, Rac and Cdc42 (all of which have been suggested to mediate the regulation of convergent extension in *Xenopus*) (Darken et al., 2002; Djiane et al., 2000; Habas et al., 2001; Heisenberg et al., 2000; Park and Moon, 2002; Sokol, 1996; Tada and Smith, 2000; Wallingford and Harland, 2001; Wallingford et al., 2000).

One of the organizer-specific transcription factors is the LIM class homeodomain protein Xlim-1 (Taira et al., 1992). The LIM domain mutant of Xlim-1, named Xlim-1/3m, or a complex of Xlim-1 and the LIM domain-binding protein Ldb1, appears to behave as an activated form of Xlim-1. Activated forms of Xlim-1 can promote the formation of a partial secondary axis in whole embryos when expressed ventrally, and can initiate expression of the organizer-specific genes *goosecoid* (*gsc*), *chordin* and *Xotx2*, in animal caps (Agulnick et al., 1996; Mochizuki et al., 2000; Taira et al., 1994; Taira et al., 1997), suggesting that Xlim-1 is involved in the functions of the organizer. Using differential screening, we searched for genes that function downstream of Xlim-1, and found that one such gene was the *Xenopus* ortholog of the mammalian *ror2* (*Xror2*), which is an orphan receptor tyrosine kinase with an immunoglobulin domain, a Frizzled-like domain, and a kringle domain in the ectodomain (Oishi et al., 1999; Rehn et al., 1998). Previous papers have reported that the *ror* gene, *cam-1*/*kin-8*, in *C. elegans* is involved in asymmetrical cell division and the migration of neural cells (Forrester et al., 1999), as well as in dauer larva formation (Koga et al., 1999), and that mouse *Ror2* is required for heart development and skeletal patterning during cartilage development (DeChiara et al., 2000; Takeuchi et al., 2000). However, the functions of the Ror family genes, *Ror1* and *Ror2*, in the early embryogenesis of vertebrates have not been elucidated. In this study, we found that *Xror2* was expressed mainly in the dorsal mesoderm and posterior neuroectoderm, where dynamic morphogenetic movements are observed (Keller et al., 1992), and that Xror2 played a role in convergent extension through the PCP pathway of Wnt signaling in *Xenopus laevis*.

## MATERIALS AND METHODS

### *Xenopus* embryos and embryo manipulation

Eggs were artificially fertilized with testis homogenates, and reared in 0.1× Steinberg's solution at 14-21°C until the desired stages were reached, following the methods of Nieuwkoop and Faber (Nieuwkoop and Faber, 1967). Animal caps were cultured in 1× MBS (Modified Barth's solution) containing 0.1% bovine serum albumin (BSA) in the presence or absence of activin A (190 pM), cycloheximide (CHX, 10 μg/ml) or dexamethasone (DEX, 10 μM).

### Construction and screening of a subtracted library

About 500 animal cap explants from embryos pre-injected with 250 pg of Xlim-1/3m mRNA or uninjected (negative control) explants were prepared at the blastula stage and cultured until the early gastrula stage (stage 10.5). Poly(A)$^+$ RNA was purified from total RNA of animal caps using the PolyATtract mRNA isolation system (Promega). cDNA synthesis and suppression PCR for creating a subtracted cDNA library were performed using the PCR-Select cDNA subtraction kit (Clontech). To avoid the concentration of cDNAs derived from injected Xlim-1/3m mRNA, 30 ng of Xlim-1/3m mRNA was added to a 2 μg poly(A)$^+$RNA pool of negative controls before synthesizing cDNA. Subtracted cDNA fragments were cloned into pT7 Blue (R) vector (Novagen) for colony hybridization with the subtracted PCR cDNA pool and the non-subtracted PCR cDNA pool. To obtain insert DNA fragments, each bacterial colony was directly subjected to PCR with a T7 promoter primer and a U-19 primer with SP6 promoter sequences. PCR products were subjected to DNA sequencing using an ABI PRISM 310 Genetic Analyser (Perkin Elmer) or used as templates for digoxigenin-labeled RNA probes for whole-mount in situ hybridization.

### Screening of a cDNA library and DNA sequencing

A *Xenopus* gastrulae cDNA library (stages 10.5 and 11.5; kindly provided by Dr B. Blumberg) was screened by plaque hybridization with PCR-amplified cDNA fragments as probes. Positive clones were sequenced for both strands with the Thermo Sequenase Cycle sequencing kit (Amersham) or a cDNA sequencing kit (Perkin Elmer) and analyzed with LONG READIR 4200 (Li-Cor) or ABI PRISM 310, respectively. Amino acid sequences were aligned using the PILEUP program of the Wisconsin Package, Version 10.0 (Genetic Computer Group, GCG, Madison, Wisconsin).

### RNA preparation and northern hybridization

Total RNA was extracted by the acid phenol method (Chomczynski and Sacchi, 1987), electrophoresed on agarose-formaldehyde gels and blotted onto a nylon membrane (Nytran, Schleicher and Shuell) (Sambrook et al., 1989). Blots were hybridized with $^{32}$P-labeled DNA probes, washed with 2× SSPE containing 0.1% SDS at 65°C, and exposed to an imaging plate and measured using a BAS 2500 (Fuji).

### Whole-mount in situ hybridization and histological studies

Whole-mount in situ hybridization was carried out according to Harland's method (Harland, 1991) with or without an automated system (Automated ISH System AIH-101, Aloka). For hemisections, rehydrated embryos were cut with a razor blade in 1× PBS, 0.1% Tween 20 before hybridization. Probes were synthesized from pBluescript II SK(–)-Xror2 (pSK–Xror2), which was the longest clone we obtained, *en2* (Hemmati-Brivanlou et al., 1991), *nrp1* (Knecht et al., 1995; Richter et al., 1990) and *XPA26* (Hikasa and Taira, 2001) using DIG or fluorescein RNA Labeling Mix (Boehringer Mannheim). BM Purple (Boehringer Mannheim), BCIP (Boehringer Mannheim) and Magenta phosphate (Sigma) were used for chromogenic reactions. Some stained embryos were embedded in paraffin wax and sectioned at widths between 10 and 15 μm.

### Plasmid constructs for mRNA injection experiments

pCS2+MT1-GR-ΔNA was constructed by inserting fragments encoding a Myc tag, the hormone-binding domain (amino acids 511-777) of the human glucocorticoid receptor (Hollenberg et al., 1993) and Xlim-1/ΔNA (Taira et al., 1994) into pCS2+AdN (Mochizuki et al., 2000). pCS2-Xror2, pCS2-Xror2-TM or pCS2-Xror2-KR were constructed by inserting a PCR fragment encoding full-length, amino acids 1-469 or amino acids 1-399, respectively, of Xror2 into pCS2+. pCS2-Xwnt5a-Myc, pCS2-Xwnt8-Myc and pCS2-Xwnt11-Myc were generated by inserting the coding regions into pCS2+MT to connect five Myc tags at their C termini. pCS2-Exfz7-FLAG was constructed by inserting PCR fragments (amino acids 1-209 of Xfz7) into pCS2+FTc, which encodes a FLAG tag at the C terminus (T. Mochizuki and M. T.). A point mutant (pCS2-Xror2-3I), small deletion mutants (pCS2-Xror2-FZΔ1 and pCS2-Xror2TM-FZΔ1) and a C-terminal FLAG-tagged construct (pCS2-Xror2KR-FLAG) were

generated using an in vitro site-directed mutagenesis system (GeneEditor, Promega). All constructs were verified by sequencing. For mRNA injection, plasmid constructs were linearized with appropriate restriction enzymes, and transcribed using the MEGAscript kit (Ambion) and a 7mG(5′)ppp(5′)G CAP analog (New England Biolabs). mRNA (20 pg/embryo) encoding nuclear β-galactosidase (nβ-gal) was co-injected as a lineage tracer, and the enzyme activity of nβ-gal was visualized using Red-Gal (Research Organics) as substrate. Some embryos stained with β-gal reaction were subjected to whole-mount in situ hybridization or embedded in paraffin wax for sectioning.

### Immunoprecipitation and western blotting

Immunoprecipitation was carried out as described previously (Djiane et al., 2000) with some modifications. Embryos were injected with mRNAs in the animal pole region at the two-cell stage. Nine injected embryos at the mid-gastrula stage were homogenized with 900 μl of extraction buffer (150 mM NaCl, 5 mM EDTA, 0.5% NP-40, 10 mM Tris-HCl pH 7.5, 2 mM PMSF, 25 μM leupeptin and 0.2 units/ml aprotinin). Cell extracts (900 μl) were incubated with anti-FLAG M2 antibody (Sigma) for 2 hours at room temperature and further incubated at 4°C for 3 hours after adding 40 μl of protein G agarose beads (Roche). Proteins attached to the beads were washed with extraction buffer four times, subjected to SDS-PAGE and blotted to an Immobilon membrane (Millipore). Blotted membranes were exposed to anti-Myc 9E10 monoclonal antibody conjugated with peroxidase (BioMol Research Lab) and were developed by ECL+plus reagents (Amersham).

## RESULTS

### The receptor tyrosine kinase gene *Xror2* is identified as a downstream gene of Xlim-1

About 15,000 clones from a subtracted cDNA library were screened using colony hybridization with subtracted and non-subtracted cDNA probes. Of them, 935 clones showed stronger signals with subtracted probes than with non-subtracted probes. To eliminate known genes that are upregulated by Xlim-1, colony hybridization was further carried out with mixed probes of *chordin*, *gsc* and *Xotx2*. Five hundred and twenty-two clones were hybridized with the mixed probes, indicating that more than half of the cDNAs upregulated by Xlim-1/3m in animal caps are *chordin*, *gsc* and *Xotx2*. The remaining 413 clones were subjected to northern blot analysis using RNAs isolated from Xlim-1/3m-injected animal caps, uninjected animal caps and the organizer region dissected from gastrula embryos. As a result, we obtained 35 independent genes that are activated by Xlim-1/3m in animal caps, and found that eight genes are expressed in the organizer region. So far, we have identified five genes as *cerberus* (Bouwmeester et al., 1996), *Xzic3* (Nakata et al., 1997), *Xotx5* (Kuroda et al., 2000; Vignali et al., 2000) (data not shown), the *Xenopus* ortholog of human *PA26* (Hikasa and Taira, 2000) and the *Xenopus* ortholog of *ror2*, referred to as *Xror2* (see below).

The longest Xror2 cDNA clone we isolated is 3924 bp long and encodes a predicted protein of 930 amino acids that is highly homologous to human and mouse Ror2, a receptor tyrosine kinase (Masiakowski and Carroll, 1992; Oishi et al., 1999) (Fig. 1A). Ror family proteins contain an immunoglobulin-like domain, a Frizzled-like domain, a kringle domain, a transmembrane domain and a tyrosine kinase domain (Masiakowski and Carroll, 1992; Oishi et al., 1999). The Frizzled-like domain of Xror2 has a motif containing 10 conserved cysteines (Fig. 1A, asterisks) that is characteristic of the ectodomain of the Wnt receptor Frizzled family (Rehn et al., 1998). However, the ligand of the Ror receptor family has not yet been identified. Within the tyrosine kinase domain, Xror2 has a predicted ATP-binding motif (GX**D**XXG–AIK) that is conserved among the Ror2 proteins but not the Ror1 proteins (GX**C**XXG–AIK) (Fig. 1A,B) (Oishi et al., 1999).

For the functional analysis of Xror2 as described below, we made five mutant constructs (Fig. 1B). Xror2-3I is a kinase domain point-mutant in which three lysines at position 504 (in the putative ATP-binding motif), 507 and 509 were all replaced with isoleucine. Xror2-TM is a kinase domain-deleted mutant in which the intracellular region, including the tyrosine kinase domain, was deleted. Xror2-KR is a putative secreted type construct that contains only the ectodomain. Xror2-FZΔ1 and Xror2TM-FZΔ1 are Frizzled-like domain-deleted mutants in which 20 amino acid residues (positions 175-194), including the second cysteine in the Xror2 Frizzled-like domain, were deleted from wild-type Xror2 and Xror2-TM, respectively.

### Xror2 is upregulated by Xlim-1 plus Ldb1 and BMP antagonists

Northern blot analyses showed that expression of *Xror2* is activated by co-expression of Xlim-1 and Ldb1, and by the BMP antagonists chordin and noggin in animal caps (Fig. 2A,B). Because the *chordin* gene is upregulated by Xlim-1 in animal caps (Fig. 2A), *Xror2* expression may be mediated by *chordin* expression. However, while *chordin* expression was reduced in animal caps injected with lower doses of Xlim-1/3m or Xlim-1 plus Ldb1 (Fig. 2A), *Xror2* expression was maintained under the same conditions, suggesting that *Xror2* expression by Xlim-1 is not solely mediated by *chordin*.

To elucidate whether or not protein synthesis is required for the activation of *Xror2* gene by Xlim-1 in animal caps, we constructed a hormone-inducible construct of an active form of Xlim-1 (GR-ΔNA) (Gammill and Sive, 1997; Tada et al., 1998; Tada and Smith, 2000; Taira et al., 1994). As shown in Fig. 2C, induction of *Xror2* expression by GR-ΔNA in the presence of DEX was inhibited by CHX, suggesting that *Xror2* expression is indirectly activated by Xlim-1 in animal caps. Conversely, in agreement with our previous report (Mochizuki et al., 2000), activation of the *gsc* gene by GR-ΔNA was not inhibited by CHX, emphasizing that *gsc* is a direct target of Xlim-1.

### Spatiotemporal expression of *Xror2* in *Xenopus* embryos

Northern blot analysis was performed to analyze the temporal expression of *Xror2*. While maternal transcripts of *Xror2* were not detected during cleavage stages, expression of *Xror2* was first detected at the early gastrula stage (stage 10), and its expression peaked from the early neurula stage (stage 13) to the mid-neurula stage (stage 15) (Fig. 2D). Up to the tailbud stage (stage 28), the expression of *Xror2* was maintained at high levels (Fig. 2D).

Whole-mount in situ hybridization showed that *Xror2* transcripts are first observed in the DMZ at the early gastrula stage (stage 10.25) with laterally expanding expression (Fig. 3A). In early gastrula embryos bisected along the midline (stage 10.25), *Xror2* transcripts were detected mainly in the dorsal mesoderm and ectoderm above the dorsal lip (Fig. 3B). As shown in Fig. 3C, the expression of both *Xror2* (left) and

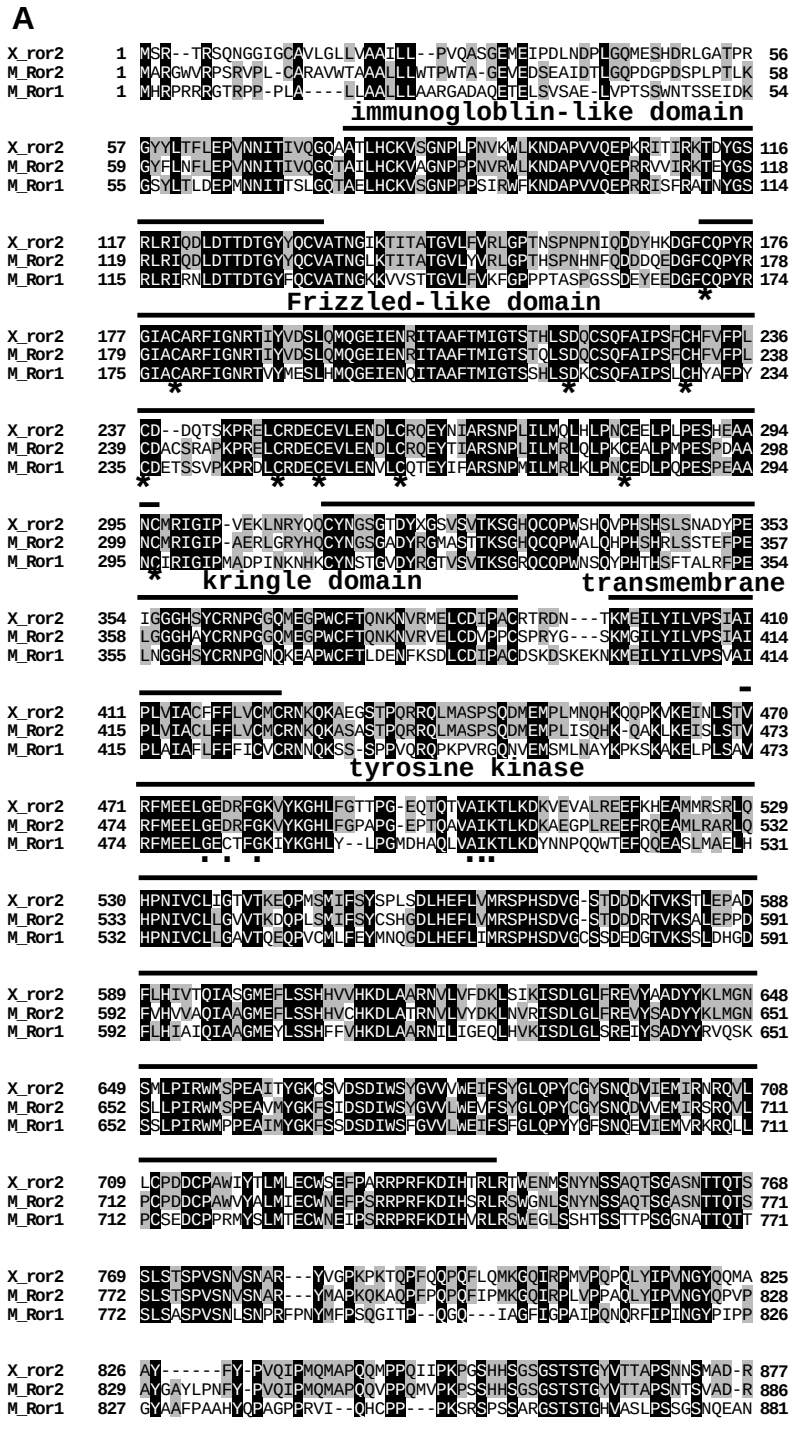


**Fig. 1.** Structure of Xror2 and its mutant constructs. (A) Comparison of predicted amino acid sequences of Xror2 and mouse Ror2 and Ror1. X_ror2, Xror2 accession no. AB087137; M_Ror2, mouse Ror2 (NM013846); M_Ror1, mouse Ror1 (NM013845). The domain structure of the Ror family is indicated by overlines with the domain names. Conserved residues shared by all or by two of them are in black or shaded boxes, respectively. Asterisks, conserved cysteines in the Frizzled-like domain; dashes, spaces for alignment; dots, predicted ATP-binding motifs. (B) Schematic diagrams of Xror2 constructs. SP, signal peptide; IG, immunoglobulin-like domain; FZ, Frizzled-like domain; KR, kringle domain; TM, transmembrane domain; TK, tyrosine kinase domain. See text for details.

*Xlim-1* (right) was observed strongly in the dorsal mesoderm and also faintly in the ventral mesoderm, overlapping with each other except for the expression of *Xror2* in the dorsal ectoderm. As gastrulation proceeded, Xror2 transcripts were intensely detected in the mesoderm and the posterior portion of the overlying dorsal ectoderm, but not in the dorsal endomesoderm (Fig. 3D).

Expression of *Xror2* in the epithelial and sensorial layers of the neuroectoderm is maintained in late gastrula to early neurula embryos (stages 12 to 14, Fig. 3E,F), whereas the expression in the mesoderm is restricted to the notochord at stage 14 (Fig. 3F). In the neuroectoderm of neurula embryos (stages 15 to 17), *Xror2* transcripts were detected with a clear border at the anterior limit of the expression domain (Fig. 3G,H). Fig. 3I shows that the anterior border of *Xror2* expression (magenta) is posterior to the midbrain-hindbrain boundary indicated by the *en2* expression (turquoise), although the lateral stripes of *Xror2* expression corresponding to the neural crest extends more anteriorly than does *en2* expression. At this stage, the transverse sections showed that *Xror2* expression was detected in both epithelial and sensorial layers of the neuroectoderm and also faintly in the notochord (Fig. 3J). At tailbud stages, the anterior border of *Xror2* expression in the neural tube became obscure, but the expression in pharyngeal arches 1 to 4 was clearly observed (Fig. 3K) (Sadaghiani and Thiebaud, 1987).

### Xror2 and its intracellular domain mutant constructs cause a shortened anteroposterior axis accompanied by head defects

If *Xror2* is a downstream gene of Xlim-1 in the organizer, it is possible that Xror2 takes part in the functions of Xlim-1. We therefore tested first whether or not ectopic expression of Xror2 in the ventral marginal zone (VMZ) initiates secondary axis

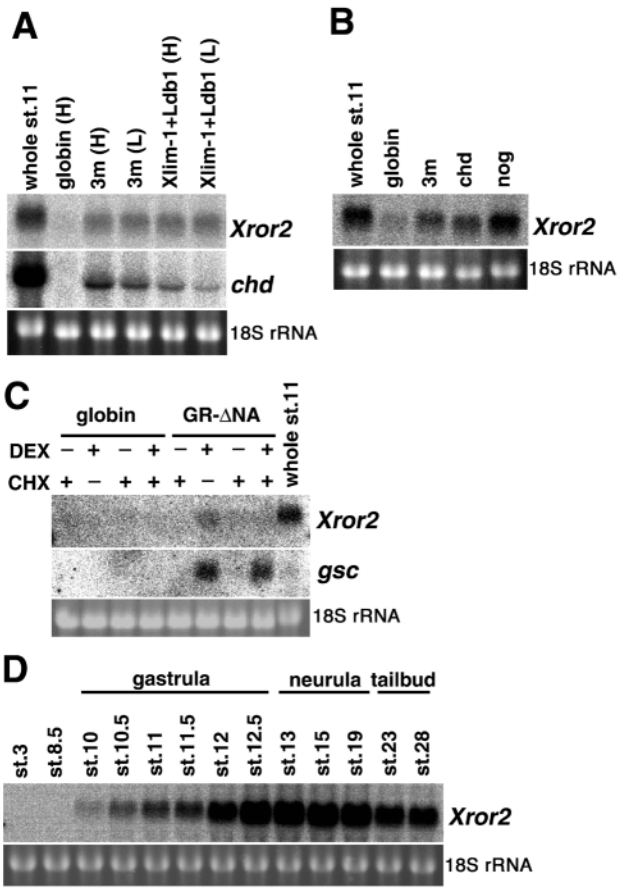


**Fig. 2.** Northern blot analysis. (A) Xlim-1/3m (3m) and Xlim-1 plus Ldb1 initiate expression of *Xror2* and *chordin* in animal caps. (B) BMP antagonists, chordin (chd) and noggin (nog), initiate expression of *Xror2* in animal caps. (C) Activation of *Xror2* expression by Xlim-1 requires protein synthesis. Animal caps (stages 8-8.5) were treated with CHX and 30 minutes later with DEX until the equivalent of stage 11. In A-C, Amounts of injected mRNA (pg/embryo): globin, 250-1000; 3m (H) or 3m, 250; 3m (L), 125; Xlim1+Ldb1(H), 250 each; Xlim1+Ldb1(L), 125 each; chordin, 250; noggin, 125; GR-ΔNA, 250. (D) Developmental expression pattern of *Xror2*. st., developmental stage; whole, whole embryos. Probes are indicated on the right-hand side. Ethidium bromide-stained 18S rRNA is for loading control.

formation. As a result, Xror2 did not elicit any apparent ectopic axis even at high doses (1-3 ng/embryo), but instead caused malformation in posterior structures (48%, *n*=159; Fig. 4B), whereas Xlim-1/3m initiated secondary axis formation (data not shown), as reported (Taira et al., 1994).

We next overexpressed Xror2 constructs in the DMZ, where putative ligands for Xror2 may exist. Embryos injected with wild-type Xror2 showed a shortened body axis with dorsal bending and abnormalities in head structures, which included one-eyed phenotypes (73%, *n*=112; Fig. 4D). The frequency of these phenotypes was reduced, but not abolished completely, when the kinase domain point mutant Xror2-3I was expressed (30%, *n*=70; Fig. 4E), implying that the kinase activity may not be essential for this phenotype. This possibility was supported by the results that phenotypes with the kinase domain deletion mutant Xror2-TM were similar to those with

**Table 1. Distribution of nβ-gal-positive cells in embryos co-injected with Xror2 constructs**

| | | | | Phenotype (%) | | | |
|---|---|---|---|---|---|---|---|
| | | | | Laterally expanded | | Medially restricted | |
| mRNA | Amount (ng/embryo) | Molar ratio | *n* | Total | Oyp | Total | Oyp |
| Globin | 1 | 2 | 58 | 7 | 2 | 93 | 9 |
| | 2 | 4 | 32 | 9 | 3 | 91 | 12 |
| Xror2 | 1 | 1 | 48 | 96 | 81 | 4 | 0 |
| | 2 | 2 | 27 | 100 | 93 | 0 | 0 |
| Xror2-3I | 1 | 1 | 49 | 67 (2*) | 47 | 33 | 2 |
| | 2 | 2 | 26 | 85 (8*) | 58 | 15 | 4 |
| Xror2-TM | 1 | 2 | 56 | 91 (57*) | 79 | 9 | 2 |
| | 2 | 4 | 30 | 100 (70*) | 83 | 0 | 0 |
| Xror2-KR | 1 | 2 | 49 | 12 | 4 | 88 | 10 |
| | 2 | 4 | 50 | 22 | 18 | 78 | 16 |

Injection of embryos was as described in Fig. 5. Phenotypes were scored based on morphological appearances and distribution of nβ-gal positive cells at stages 13-14 (see Fig. 5A-F). The data were obtained from two separate experiments. *n*, number of scored embryos; Oyp, open yolk plug phenotype.
*Embryos with condensation of pigmented cells in nβ-gal-positive region.

Xror2-3I in terms of short stature and head defects (50%, *n*=58; Fig. 4F). In contrast to Xror2-3I and Xror2-TM, Xror2-KR showed much weaker phenotypes, even at a twofold molar ratio to that of wild-type (5%, *n*=93; Fig. 4G), suggesting that the phenotypes with wild-type, Xror2-3I and Xror2-TM are not due to the depletion of its putative ligands. Thus, these data suggest that the membrane-anchored ectodomain of Xror2 per se appears to have some role for cell-cell communications, which could be enhanced by its kinase activity.

### Xror2 and its intracellular domain mutants interfere with convergent extension during gastrulation and affect neural plate closure during neurulation

To examine whether overexpression of Xror2 constructs on the dorsal side leads to abnormalities in morphogenetic movements, nβ-gal mRNA was co-injected as a tracer into one blastomere on the right side at the four-cell stage. As shown in Fig. 5A, globin-expressing control cells were restricted in their distribution along the midline in the trunk region as a result of normal convergent extension, and not in the head region as expected. By contrast, cells expressing Xror2, Xror2-3I or Xror2-TM expanded laterally on the right side of both the mesoderm and ectoderm layers (Fig. 5B-D). The ability of wild-type Xror2 to interfere with convergent extension was higher than that of the Xror2 kinase domain mutants (Xror2-3I and Xror2-TM) at similar molar levels (Table 1). Xror2-KR seemed to slightly affect convergent extension at higher doses, but most Xror2-KR-expressing embryos showed normal movements, similar to globin-expressing embryos (Fig. 5A,E; Table 1). When open yolk plug phenotypes were observed at a low frequency in embryos expressing globin and Xror2-KR, nβ-gal-positive cells converged to the midline at a significant frequency (Fig. 5F; Table 1), implying that convergent extension and yolk plug closure are separable events (see also Fig. 5B′).

We also noticed that Xror2-TM initiates the condensation of pigmented cells at early neurula stages in nβ-gal-positive

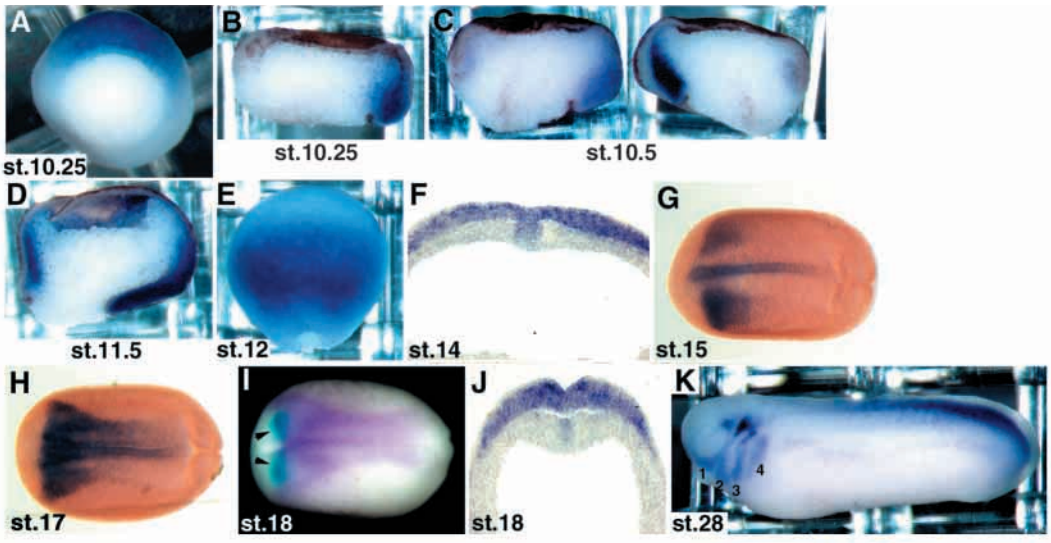


**Fig. 3.** Localization of *Xror2* transcripts visualized by whole-mount in situ hybridization. (A) Vegetal view of the gastrula (dorsal is upwards). (B) Hemisectioned early gastrula (animal is upwards; dorsal towards the right). Embryos were bisected sagittally before whole-mount in situ hybridization. (C) Comparison of expression domains between *Xror2* (left panel) and *Xlim-1* (right panel) at the gastrula stage. Embryos were bisected into left and right halves and subjected to in situ hybridization for *Xlim-1* and *Xror2*, respectively. Dorsal is towards the right (*Xror2*) or the left (*Xlim-1*). (D) Late gastrula bisected sagittally (animal is upwards; dorsal towards the right). (E) Dorsal view of late gastrula (anterior is upwards). (F) Transverse section of stained embryos. *Xror2* transcripts are detected in the notochord and neuroectoderm. (G,H) Dorsal view of mid-neurula embryos cleared with benzyl benzoate/benzyl alcohol. *Xror2* transcripts were detected in the notochord and neuroectoderm with a clear anterior limit in the neuroectoderm. (I) Double whole-mount in situ analysis of *Xror2* (magenta) and *en2* (turquoise). Arrowheads, *en2* expression. (J) Transverse section of neurula embryos. (K) Lateral view of tailbud-stage embryos. Numbers 1, 2, 3 and 4 indicate *Xror2* expression in mandibular crest, hyoid crest, anterior branchial crest and posterior branchial crest segments, respectively. st., developmental stages.

regions of dorsal ectoderm (Fig. 5D, black arrows and arrowheads; Table 1; see also Fig. 6C). This activity differs from that of wild-type and Xror2-3I. At the late neurula stage (stage 18), Xror2 or Xror2-3I mRNA-injected embryos still failed to close the neural plate in nβ-gal-positive regions (Fig. 5H,I, white arrows) compared with globin- and Xror2-KR-expressing embryos (Fig. 5G,K; Table 2), whereas Xror2-TM mRNA-injected embryos showed the closing neural plate with pigmented cells (Fig. 5J, white arrowheads; Table 2). These data imply that Xror2 has distinct roles in neurulation of the pigmented epithelial layer and the sensorial layer.

To examine whether or not the Frizzled-like domain of Xror2 is involved in its functional activities, we constructed mutants with a small deletion in the Frizzled-like domain, based on the report that the same mutation in *Drosophila* Frizzled 2 inhibits Wnt binding (Hsieh et al., 1999) (Fig. 1B). We observed that Xror2-FZΔ1 and Xror2TM-FZΔ1 produced much less inhibition on convergent extension and neural plate closure during gastrulation and neurulation in comparison with Xror2 and Xror2-TM, respectively (Table 3 and data not shown). These results indicate that the effect of Xror2 on morphogenetic movements is significantly dependent on its Frizzled-like domain, raising the possibility that Xror2 might interact with a Wnt pathway.

## Xror2 does not affect gene expression of molecular markers for neural and notochordal differentiation

To test whether wild type and mutant versions of Xror2 affect the cell fate of neural tissue and the notochord, we analyzed expression of *nrp1* as a pan-neural marker (Knecht et al., 1995) and *XPA26* as a notochord marker (Hikasa and Taira, 2001) in embryos co-injected with Xror2 constructs and nβ-gal mRNA. In Xror2- (Fig. 6B,E), Xror2-3I- (not shown) or Xror2-TM- (Fig. 6C,F) expressing embryos, nβ-gal-positive cells overlapped with *nrp1* and *XPA26* expression, similar to control cells (Fig. 6A,D). It should also be noted that the notochord is stacked near the unclosed blastopore and failed to elongate anteriorly in Xror2- (Fig. 6E), Xror2-3I- (not shown) and Xror2-TM- (Fig. 6F) expressing embryos. This is clearly

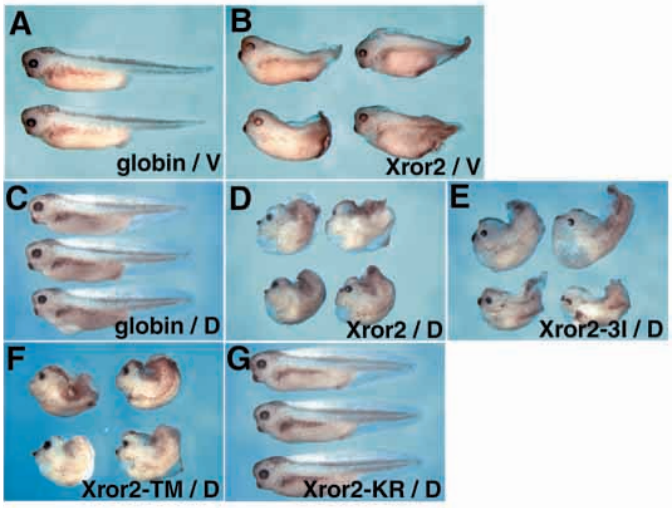


**Fig. 4.** Overexpression of Xror2 and its mutant constructs causes a shortened anteroposterior axis. Two blastomeres of four-cell stage embryos were injected in the ventral (/V) or dorsal (/D) equatorial region with mRNAs (1 ng/embryo) as indicated (A-G). Injected embryos were cultured until stage 38. (A,C) Globin mRNA-injected control embryos. (B) Ventral overexpression of Xror2 causes malformation in posterior structures. (D-F) Dorsal overexpression of Xror2 and kinase domain mutants (Xror2-3I, Xror2-TM) causes a shortened body axis with dorsal bending and abnormalities in head structures, including one-eyed phenotypes. (G) Xror2-KR shows much weaker phenotypes than the other constructs of Xror2.

**Table 2. Effects of Xror2 constructs on neural plate closure**

| mRNA | Amount (ng/embryo) | Molar ratio | *n* | Phenotype (%) Failure of neural plate closure | Neural groove like structure | Normal |
|---|---|---|---|---|---|---|
| Globin | 1 | 2 | 48 | 2 | 0 | 83 |
| | 2 | 4 | 27 | 4 | 0 | 89 |
| Xror2 | 1 | 1 | 49 | 80 | 0 | 4 |
| | 2 | 2 | 25 | 76 | 0 | 0 |
| Xror2-3I | 1 | 1 | 45 | 22 | 9 | 22 |
| | 2 | 2 | 29 | 48 | 3 | 31 |
| Xror2-TM | 1 | 2 | 60 | 2 | 70 | 12 |
| | 2 | 4 | 30 | 0 | 90 | 7 |
| Xror2-KR | 1 | 2 | 52 | 2 | 4 | 79 |
| | 2 | 4 | 46 | 2 | 2 | 63 |

Injection of embryos was as described in Fig. 5. Phenotypes were scored based on morphological appearances and distribution of nβ-gal-positive cells at stage 18-19 (see Fig. 5G-K). The data were obtained from two separate experiments. *n*, number of scored embryos.

different from the open yolk plug phenotype caused by the inhibition of mesoderm formation by a dominant-negative FGF receptor, in which two columns of notochord extend posteriorly around the blastopore (Isaacs et al., 1994). Moreover, we observed that these Xror2 constructs do not affect the staining with the muscle-specific antibody 12/101 (data not shown). Thus, we conclude that the failure of convergent extension caused by Xror2 constructs was not due to changes in cell fate.

## Effects of Xror2 constructs on morphogenetic movements in animal caps

Elongation of animal caps by treatment with activin provides a useful model system for analyzing convergent extension in *Xenopus* (Djiane et al., 2000; Tada and Smith, 2000). Xror2, Xror2-3I and Xror2-TM strongly suppressed elongation of animal caps by activin (Fig. 7A-D), consistent with the observations in whole embryos (Fig. 5). Compared with globin-expressing animal caps, elongation was slightly reduced by Xror2-KR, suggesting that Xror2-KR has a weak activity that is not apparent in whole embryos (Fig. 7A,E). In Xror2-TM-expressing animal caps treated with activin, neural groove-like structures with pigmentation were also observed (Fig. 7D, arrowheads), as has been seen in Xror2-TM-expressing whole embryos (Fig. 5J).

Previous studies of Xwnt11 and Dishevelled have shown that both wild-type and a dominant-negative form of these proteins have activity to inhibit the morphogenetic movements of animal caps or DMZ explants, but coexpression of both proteins can offset the phenotype from either of them (Tada and Smith, 2000; Wallingford et al., 2000). These phenomena imply that opposite effects on cell polarity can eventually show similar phenotypes. To elucidate whether or not this relationship is applied to wild-type Xror2 and Xror2-TM, we co-expressed these constructs in animal caps treated with activin. Either wild-type Xror2 or Xror2-TM alone at low doses moderately inhibited elongation of activin-treated animal caps (Fig. 7F,G,I), whereas co-expression of these two constructs inhibited the elongation more strongly (Fig. 7H). These results indicate that wild-type and Xror2-TM have the same activity in terms of interference with the morphogenetic movements.

## Functional interactions between Xror2 and Wnt signaling components

As mentioned above, the results from overexpression of the Frizzled-like domain mutants (Table 3) implied the possibility that the activity of Xror2 could be involved in Wnt signaling. Both Xwnt11 and Xfz7 have been shown to regulate convergent extension (Djiane et al., 2000; Ku and Melton, 1993; Tada and Smith, 2000), and exhibit significant overlapping expression with Xror2 in the dorsal marginal zone (Fig. 3). To assess involvement of Xror2 in Wnt signaling, we first examined the effects of co-expression of Xror2, Xwnt11 and Xfz7 on convergent extension (Table 4). A low dose of Xror2 or Xwnt11 alone (25 or 50 pg/embryo, respectively) had a very small effect on convergent extension, but a combination of Xror2 plus Xwnt11 showed synergistic effects, which were higher than those of twofold doses of either alone. Interestingly, such synergy was also observed between Xror2 and Xfz7. Moreover, co-expression of the three proteins Xror2, Xwnt11 and Xfz7 exerted stronger effects than those

**Table 3. Frizzled-like domain mutants of Xror2 have little effect on convergent extension**

| mRNA | Amount (ng/embryo) | *n* | Phenotype (%) Laterally expanded Total | Laterally expanded Oyp | Medially restricted Total | Medially restricted Oyp | Number of experiments |
|---|---|---|---|---|---|---|---|
| Globin | 0.5 | 37 | 8 | 5 | 92 | 0 | 1 |
| | 1 | 75 | 7 | 0 | 93 | 0 | 2 |
| Xror2 | 0.5 | 35 | 89 | 60 | 11 | 0 | 1 |
| | 1 | 74 | 99 | 84 | 1 | 0 | 2 |
| Xror2-FZΔ1 | 0.5 | 36 | 6 | 0 | 94 | 0 | 1 |
| | 1 | 72 | 17 | 8 | 83 | 0 | 2 |
| Xror2-TM | 1 | 40 | 98 (38*) | 98 (38*) | 2 (2*) | 0 | 1 |
| Xror2-TM-FZΔ1 | 1 | 43 | 16 | 12 | 84 | 0 | 1 |

Injection of embryos and scoring of phenotypes were as described in Fig. 5 and Table 1, respectively. *n*, number of scored embryos; Oyp, open yolk plug phenotype.

*Embryos with condensation of pigmented cells in nβ-gal-positive region.

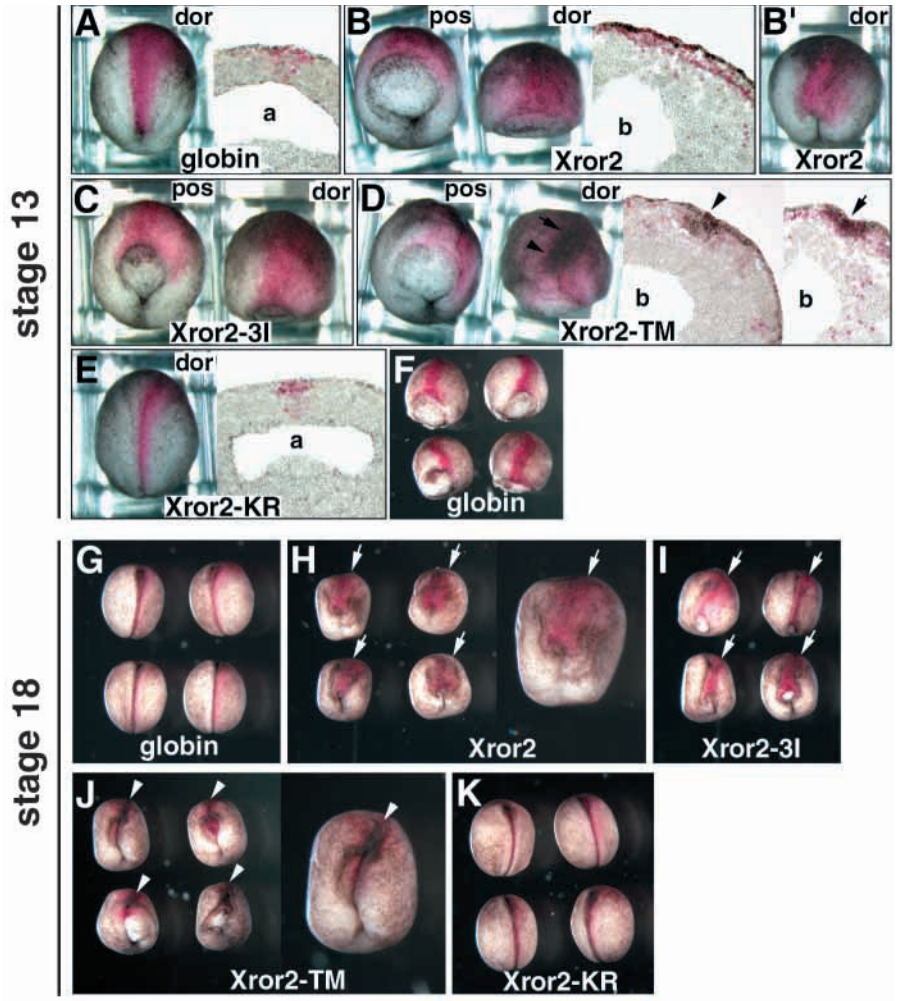


**Fig. 5.** Analysis of convergent extension and neural plate closure after overexpression of Xror2 constructs. mRNA (1 ng/embryo) of globin (A,G), Xror2 (B,B′,H), Xror2-3I (C,I), Xror2-TM (D,J) or Xror2-KR (E,K) as indicated was injected together with nβ-gal mRNA (20 pg/embryo) for a tracer into one blastomere on the dorsoanimal and right side at the four-cell stage, and subjected to β-gal staining (red). (A-F) Stage 13 embryos; dor, dorsal view; pos, posterior view; a, archenteron; b, blastocoel. (G-K) Stage 18 embryos (dorsal view; anterior is upwards). (A) Globin-expressing control embryos and a section. As a result of normal convergent extension, nβ-gal-positive cells were restrictedly distributed along the midline in the trunk region (see also G). (B,B′) Xror2-expressing embryos (and section in B) with and without open yolk plug, respectively. nβ-gal-stained cells were laterally expanded on the right side of both mesoderm and ectoderm layers as a result of the inhibition of convergent extension. (C) Xror2-3I-expressing embryos showing weaker phenotypes than did wild type. (D) Xror2-TM-expressing embryos. nβ-gal-stained cells were laterally expanded, and condensation of pigmented cells was observed in nβ-gal-positive regions of ectoderm, as indicated by the arrow and arrowhead, each of which show the corresponding position in a whole embryo (second panel from the left) and in sections (third or fourth panel). When nβ-gal-positive cells were laterally expanded, the archenteron was not formed (B,D). (E) Xror2-KR-expressing embryo and section showing normal-looking cell movements. (F) Globin-expressing embryos with open yolk plug. In a few embryos that had open yolk plugs as a result of overexpressing globin, nβ-gal-positive cells still converged to the midline. (G) Globin-expressing embryos at stage 18. nβ-gal-positive cells were restrictedly distributed along the midline in the trunk region. (H) Xror2-expressing embryos which failed to close the neural plate in nβ-gal-positive regions (white arrows). Right panel, higher magnification. (I) Xror2-3I-expressing embryos showing weaker phenotypes than those of wild-type Xror2-expressing embryos. (J) Xror2-TM-expressing embryos. Expression of Xror2-TM resulted in neural groove-like formation with pigmented cells (white arrowheads). Right panel shows a higher magnification. (K) Xror2-KR-expressing embryos. Xror2-KR does not have apparent phenotypes, similar to globin control.

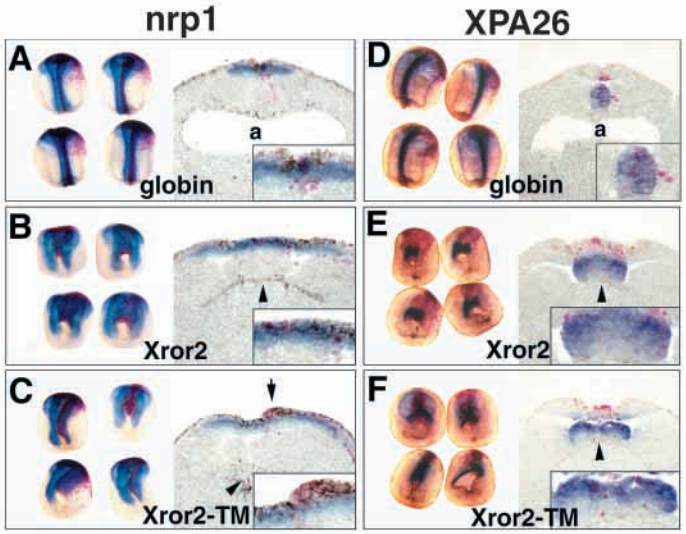


**Fig. 6.** Wild-type and its intracellular mutants of Xror2 affect convergent extension of neural tissue and notochord but not cell differentiation markers. Embryos were injected with mRNA as indicated, and were subjected at stage 18 to β-gal staining (red) and whole-mount in situ hybridization with pan-neural marker *nrp1* (A-C) or notochord marker *XPA26* (D-F) as probes. Right panel, sections of stained embryos; inset, higher magnification of neural tissue or notochord region. Expression domains of *nrp1* were widened laterally and shortened anteriorly in Xror2- (B) and Xror2-TM- (C) expressing embryos, compared with globin-expressing embryos (A). *nrp1* expression was not inhibited in nβ-gal-positive regions of Xror2- (B) and Xror2-TM- (C) expressing embryos (right panel, inset). Expression domains of *XPA26* failed to elongate anteriorly and were located near the blastopore in Xror2- (E) and Xror2-TM- (F) expressing embryos, compared with those expressing globin (D) (left panel). Areas of *XPA26* expression domains on section are much larger in Xror2- (E) and Xror2-TM- (F) expressing embryos than in globin-expressing embryos (D) (right panel, inset), whereas there is no inhibition of XPA26 expression in nβ-gal-positive regions. a, archenteron; arrowhead, collapsed archenteron; arrow, thickened pigmented epithelial layers of the ectoderm.

**Table 4. Xror2 can synergize with Xfz7 and Xwnt11 to inhibit convergent extension**

| mRNA | Amount (pg/embryo) | *n* | Phenotype (%) Laterally expanded Total | Laterally expanded Oyp | Medially restricted Total | Medially restricted Oyp | Number of experiments |
|---|---|---|---|---|---|---|---|
| Globin | 200 | 242 | 6 | 3 | 94 | 2 | 6 |
| Xror2 | 25 | 181 | 17 | 3 | 83 | 1 | 4 |
| | 50 | 121 | 31 | 12 | 69 | 2 | 2 |
| Xwnt11 | 50 | 98 | 4 | 3 | 96 | 0 | 2 |
| | 100 | 60 | 17 | 8 | 83 | 2 | 1 |
| Xfz7 | 10 | 92 | 21 | 8 | 79 | 0 | 2 |
| | 25 | 98 | 59 | 27 | 41 | 1 | 2 |
| Xror2+Xwnt11 | 25+50 | 112 | 56 | 15 | 44 | 2 | 2 |
| Xror2+Xfz7 | 25+10 | 149 | 83 | 37 | 17 | 0 | 2 |
| Xror2+Xwnt11 | 10+20 | 86 | 24 | 21 | 76 | 1 | 2 |
| Xror2+Xfz7 | 10+4 | 85 | 24 | 12 | 76 | 1 | 2 |
| Xfz7+Xwnt11 | 4+20 | 85 | 22 | 13 | 78 | 5 | 2 |
| Xror2+Xwnt11+Xfz7 | 10+20+4 | 89 | 90 | 74 | 10 | 0 | 2 |

Injection of embryos and scoring of phenotypes were as described in Fig. 5 and Table 1, respectively. *n*, number of scored embryos; Oyp, open yolk plug phenotype.

of any two of them, suggesting that Xror2, Xwnt11 and Xfz7 cooperate to function in Wnt signaling for convergent extension (Table 4).

Synergistic effects of Xror2, Xwnt11 and Xfz7 raised the possibility that Xror2 activates a PCP pathway of Wnt signaling, which has been suggested to involve the Rho family GTPase Cdc42. A dominant-negative Cdc42 mutant ($Cdc42^{T17N}$) has been shown to offset the inhibitory effect of Xwnt11 or Xfz7 on activin-induced elongation of animal caps (Djiane et al., 2000). We therefore tested whether inhibition of convergent extension by Xror2 was also rescued by $Cdc42^{T17N}$ using animal cap assays. As shown in Fig. 8, inhibition of activin-induced elongation by Xror2 was rescued by co-expression of $Cdc42^{T17N}$ to some extent (Fig. 8A,B,F), similar to that in the case of Xwnt11 and $Cdc42^{T17N}$ (Fig. 8C,D,F). Interestingly we also noticed that the inhibition of elongation by Xror2 and Xwnt11 is more effectively reversed by $Cdc42^{T17N}$ in unpigmented cells rather than in pigmented cells. These results suggest that the signal mediated by Xror2 activates Cdc42 through a PCP pathway of Wnt signaling, leading to inhibition of convergent extension.

## Physical interactions of Xror2 and Xwnt11

The existence of the Frizzled-like domain in Xror2, and the synergism between Xror2 and Xwnt11 implied possible

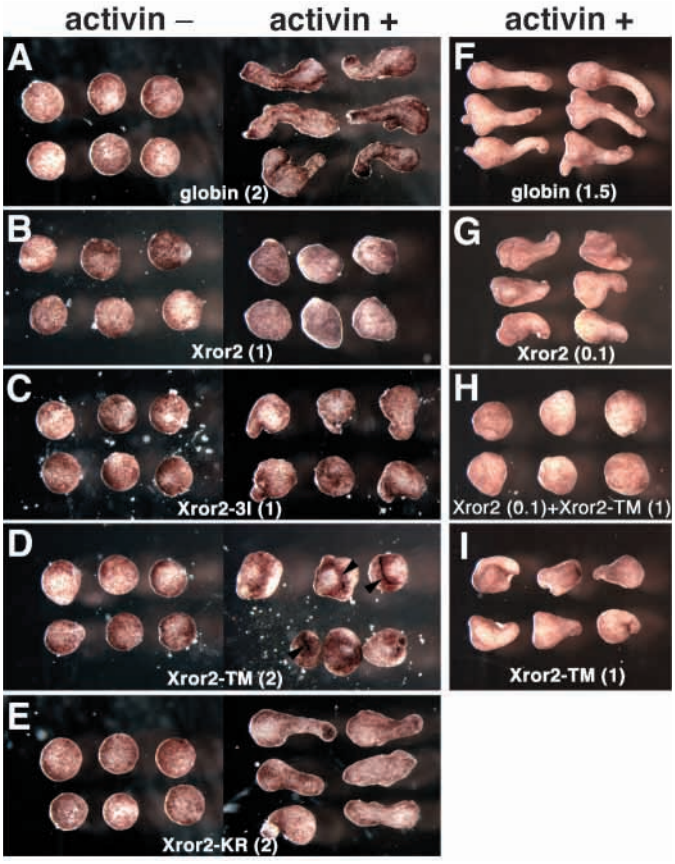


**Fig. 7.** Effects of Xror2 and its mutant constructs on morphogenetic movements of animal caps stimulated with activin. Two-cell stage embryos were injected with mRNAs of globin (A,F), Xror2 (B,G), Xror2-3I (C), Xror2-TM (D,I), Xror2-KR (E) or a mixture of Xror2 and Xror2-TM (H) in the animal pole region of both blastomeres. Doses of injected mRNA (ng/embryo) are indicated in parentheses. Note that doses of mRNA used in F-I are lower than in A-E. Animal caps (stages 8-8.5) were treated with (right panels in A-E; F-I) or without (left panels in A-E) activin A as indicated and cultured until sibling stage 18. A-E and F-I are separate experiments. Activin treatment initiated elongation of control animal caps (A,F). Xror2 (B), Xror2-3I (C) and Xror2-TM (D) suppressed elongation of animal caps by activin. In Xror2-TM-expressing animal caps, a neural groove-like structure with pigmented cells (black arrowheads) was observed in activin-treated ones. Elongation of Xror2-KR-expressing animal caps treated with activin was slightly reduced (E), compared with globin-expressing animal caps. Xror2 or Xror2-TM with lower doses show moderate inhibition of activin-induced elongation of animal caps (G,I). Co-expression of Xror2 and Xror2-TM shows cumulative effects on the inhibition of activin-induced elongation, indicating that wild-type and Xror2-TM do not compete with each other (H).

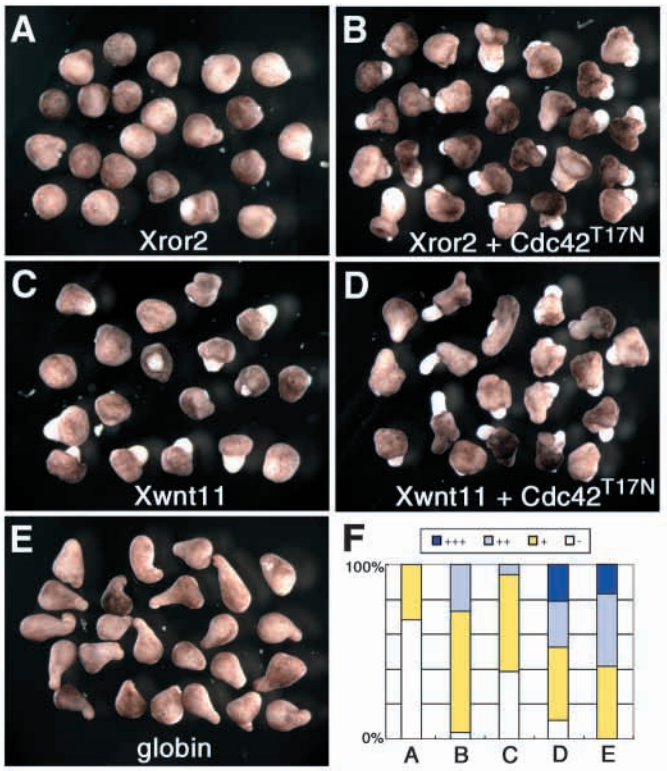


**Fig. 8.** Effects of Xror2 on activin-induced elongation of animal caps can be cancelled by $Cdc42^{T17N}$, a dominant-negative Cdc42 mutant. mRNA injection and animal cap assay were performed as described in Fig. 7. All animal caps were treated with activin A. (A) Xror2 inhibits elongation of animal caps by activin. (B) Co-expression of $Cdc42^{T17N}$ with Xror2 partially rescues the extent of explant elongation. (C) Xwnt11 inhibits elongation of animal caps. (D) Co-expression of $Cdc42^{T17N}$ with Xwnt11 partially rescues explant elongation. (E) Globin-expressing animal caps (negative control) treated with activin show elongation. Amounts of mRNA (ng/embryo):Xwnt11, 0.5; $Cdc42^{T17N}$, 0.6; Xror2, 0.2; globin, 1. (F) Summary of activin-induced elongation assay. The extent of animal cap elongation induced by activin (A-E) was classified by blind scoring as follows. –, no elongation; +, weak elongation; ++ moderate elongation; +++, strong elongation.

physical interactions between them. Using co-immunoprecipitation experiments with epitope-tagged proteins, we found that Xwnt11-Myc, Xwnt5a-Myc and Xwnt8-Myc all were co-immunoprecipitated with FLAG-tagged Xror2-KR (Xror2KR-FLAG), similar to the FLAG-tagged ectodomain of Xfz7 (Exfz7-FLAG) (Fig. 9) (Djiane et al., 2000). This data suggests that the ectodomain, most likely the Frizzled-like domain, of Xror2 can interact with several Xwnt proteins, further emphasizing the possibility that Xror2 acts together with Xwnt11 for the PCP pathway of Wnt signaling in the regulation of convergent extension.

## DISCUSSION

### Gene regulation of *Xror2* by Xlim-1 and developmental expression patterns

During the screening of a subtracted cDNA library for candidate target genes of Xlim-1, we noticed that a large percentage of the genes that are upregulated by Xlim-1/3m in animal caps are co-expressed with *Xlim-1* in the Spemann organizer region and/or later in the notochord. Those genes included *cerberus*, *Xotx5*, *Xzic3*, *XPA26* and *Xror2*, as well as the previously identified known genes, *gsc*, *chordin* and *Xotx2* (Agulnick et al., 1996; Mochizuki et al., 2000; Taira et al., 1994). This result validates our approach towards identifying candidate target genes of Xlim-1. However, *Xror2* may not be a direct target gene of Xlim-1, as *Xror2* expression induced by Xlim-1 in animal caps requires protein synthesis (Fig. 2C). These data suggest that activation of *Xror2* expression by Xlim-1 is mediated by its downstream genes such as *chordin* (Fig. 2B). Another possibility that still remains, is that Xlim-1 directly activates the *Xror2* gene together with a labile factor whose level is lowered by CHX treatment.

From the gastrula to the neurula stages, *Xror2* is expressed in the involuting mesoderm and neural plate posterior to the midbrain-hindbrain boundary (Fig. 3), where convergent extension occurs (Keller et al., 1992). Furthermore, *Xror2* is probably expressed in migrating neural crest cells in the pharyngeal arches at tailbud stages (Fig. 3K). These *Xror2* expression patterns imply a role of Xror2 in morphogenetic cell movements and cell migration. Functional analyses support this possibility as discussed below.

### Roles of Xror2 in convergent extension and neural plate closure

Using mRNA injection experiments, we found that: (1) wild-type Xror2 as well as its kinase domain mutants, Xror2-TM and Xror2-3I, cause disruption of convergent extension in whole embryos and also in animal caps treated with activin, whereas the secreted type construct Xror2-KR has a much weaker activity; (2) Xror2-TM does not antagonize wild-type Xror2 in terms of the inhibition of activin-induced elongation in animal caps; and (3) Xror2-TM initiates condensation of pigmented cells in the closing neural plate, whereas wild-type Xror2 and Xror2-3I inhibit neural plate closure and neural groove formation. These data suggest that Xror2 has roles in convergent extension and neural plate closure. Furthermore, inhibition of convergent extension by Xror2 does not appear to be dependent on the kinase domain, but dependent on the ectodomain attached to the transmembrane region. This raises the possibility that the ectodomain of Xror2 has a significant function without the tyrosine kinase domain, when it exists on the cell membrane. This possibility is consistent with the phenotype of *C. elegans ror* mutants. The *C. elegans ror* gene, *cam-1*, is required for asymmetric cell division, cell migration and axon outgrowth of a specific type of neuronal cell, and a truncated kinase domain mutant shows only subtle effects on cell migration and partial effects on asymmetric cell division. Moreover, the defects of cell motility in null *ror* mutants can be rescued by kinase-domain point-mutated constructs of the *ror* gene (Forrester et al., 1999). Thus, the ectodomain of both Xror2 and *C. elegans* Ror appears to have a function similar to that of wild-type.

Kinase-independent functions of receptor kinases have been described not only for *C. elegans* Ror, but also for other receptor tyrosine kinases. For example, the ectodomain of MuSK (muscle-specific kinase), related to the Ror family, mediates clustering of synaptic components via binding of agrin, the MuSK ligand (Apel et al., 1997). In the case of

Xror2, how can the ectodomain itself have functions without transducing signals via its kinase activity? One can speculate that Xror2 works as a cell adhesion molecule, or interacts with some membrane-anchored protein that is involved in convergent extension. Still, it should be noted that the activities of kinase domain mutants Xror2-3I and Xror2-TM, are weaker than those of wild-type Xror2 (Tables 1, 2), suggesting that the tyrosine kinase activity of Xror2 does have some function in modulating convergent extension.

It has been reported that neurulation takes place through two distinct processes of cell movements in *Xenopus* (Davidson and Keller, 1999). The first visible morphogenetic cell movements in neurulation result in neural fold fusion, in which superficial neural cells apically contract and roll the neural plate to form the neural groove. After neural fold fusion, medial migration of neural cells in a lateral sensorial layer occurs to form the dorsal tube. During these processes, we found that wild-type and 3I mutant proteins inhibit both neural fold fusion and convergent extension of medially migrating sensorial layer cells, whereas Xror2-TM inhibits convergent extension of sensorial cells but appears not to inhibit neural groove formation (Fig. 5D, Fig. 6C, Table 1). These data suggest that the intracellular region of Xror2 is involved in the regulation of the neural fold fusion of the epithelial layer.

### Xror2 and Wnt signaling

In *Drosophila*, PCP signaling through Frizzled requires the activity of a putative four transmembrane protein, Stbm and Dishevelled, and activates the small GTPase RhoA and JNK (Adler et al., 2000; Axelrod et al., 1998; Boutros and Mlodzik, 1999; Eaton et al., 1996; Strutt et al., 1997; Taylor et al., 1998; Winter et al., 2001; Wolff and Rubin, 1998). *Xenopus* PCP signaling-related genes such as a class of Xwnt11, Xfz7, Stbm, Dishevelled, Daam1 and small GTPases have been suggested to regulate convergent extension during gastrulation (Darken et al., 2002; Djiane et al., 2000; Habas et al., 2001; Park and Moon, 2002; Sokol, 1996; Tada and Smith, 2000; Wallingford and Harland, 2001; Wallingford et al., 2000). Interestingly, Xror2 has a Frizzled-like domain in the extracellular region, which is expected to interact with Wnt proteins (Rehn et al., 1998). With regard to interactions between Xror2 and Wnt signaling, our functional analyses have led to the following conclusions: (1) Xror2 has the activity to affect convergent extension, as do Xwnt11 and Xfz7; (2) the activity of Xror2 depends on its Frizzled-like domain and can be synergistic with Xwnt11 and Xfz7; (3) the inhibitory effect of Xror2 on elongation of activin-treated animal caps is modestly rescued by a dominant-negative Cdc42 mutant; and (4) the ectodomain of Xror2 can bind to Xwnt11 and Xwnt5a. These results suggest that Xror2 is involved in the non-canonical Wnt signaling for the PCP pathway. Although the rescue of the animal cap elongation by a dominant-negative Cdc42 is weak, this may be explained by the involvement of other small GTPases such as Rho and Rac in convergent extension, which have been suggested by Habas et al. (Habas et al., 2001). In addition, it is also conceivable that Xror2 stimulates the Wnt signaling through interaction with Frizzled, a seven transmembrane receptor, and perhaps Stbm, a four transmembrane protein on the plasma membrane. These possibilities are based on our findings of synergy between Xror2 and Xfz7, and the reported observations that MuSK interacts with the ligand agrin (Glass et al., 1996), the four transmembrane protein acetylcholine receptor (Fuhrer et al., 1999; Fuhrer et al., 1997), and the cytoplasmic protein rapsyn through a putative transmembrane intermediate (Apel et al., 1997) to stimulate clustering of acetylcholine receptors in the postsynaptic membrane. It is therefore tempting to speculate that Xror2 mediates or modifies the PCP signaling by complex formation with Wnt11 and the transmembrane proteins Xfz7 and Stbm to regulate convergent extension.

A loss-of-function study of Xfz7 by a morpholino approach has shown that Xfz7-depletion leads to disruption of tissue separation between mesoderm and ectoderm without affecting convergent extension (Winklbauer et al., 2001). Because Xfz8, which is closely related to Xfz7, has also been shown to be expressed at the DMZ and to affect convergent extension (Deardorff et al., 1998; Itoh et al., 1998; Wallingford et al., 2001), there might be functional redundancy between Xfz7 and Xfz8 in convergent extension.

### Comparison of *Xror2* with mammalian *Ror* genes

In mice, targeted gene disruption of *Ror2* has been shown to lead to skeletal abnormalities with endochondrally derived foreshortened or misshapen bones (DeChiara et al., 2000;

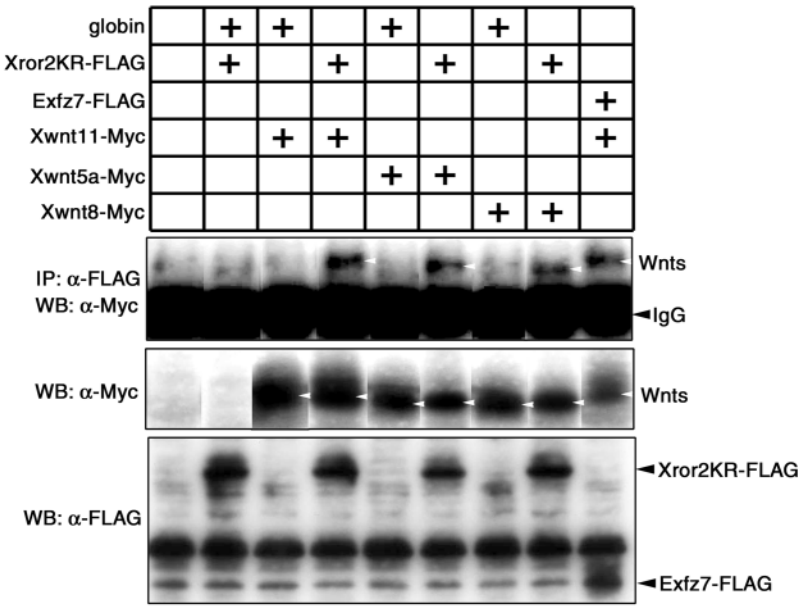


**Fig. 9.** Xror2 can associate with Xwnt11, Xwnt5a and Xwnt8. Proteins were extracted from embryos injected with mRNA as indicated, immunoprecipitated (IP) with anti-FLAG antibody, and subjected to western blotting (WB) using anti-Myc antibody (top). The equivalent amounts of proteins generated from injected mRNAs were confirmed by western blotting of lysates using anti-Myc (middle) or anti-FLAG (bottom) antibody. White arrowheads, Wnt proteins. Amounts of mRNA (ng/embryo): globin, 1 (with Xror2KR-FLAG) or 2; Xror2KR-FLAG, 2; Exfz7-FLAG, 0.5; Xwnt11-Myc, 0.5 (with Exfz7-FLAG) or 1; Xwnt5a-Myc, 1; Xwnt8-Myc, 1.

Takeuchi et al., 2000), and these phenotypes are significantly similar to those of mice disrupted with Wnt5a (Yamaguchi et al., 1999). In humans, heritable dominant mutations in the *ROR2* gene cause brachydactyly type B, in which the thumbs and big toes are spared (Oldridge et al., 2000). These data suggest that mammalian Ror genes play roles in skeletal patterning and limb development in late embryogenesis.

In *Xenopus*, our results indicate that Xror2 has roles in morphogenetic movements during gastrulation and neurulation at early developmental stages without influencing cell fates. Although mouse *Ror2* is expressed in the primitive streak (Matsuda et al., 2001), which corresponds to the dorsal mesoderm of *Xenopus*, and has the same effect as Xror2 on convergent extension when dorsally overexpressed in *Xenopus* embryos (data not shown), it is not known whether or not Ror2 functions in morphogenetic movements in mice. As cell movements in gastrulation and neurulation appear to be different between amphibians and higher vertebrates, Xror2 may have unique functions in convergent extension during gastrulation and neurulation in amphibians. Nevertheless, our data provide the first evidence that Ror2 plays a role in morphogenetic movements in relation to the PCP signaling pathway of Wnt in vertebrates, and that the ligand of Ror2 is Wnts.

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