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# Visualizing Long-Range Movement of the Morphogen Xnr2 in the *Xenopus* Embryo

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## Summary

**One way in which cells acquire positional information during embryonic development is by measuring the local concentration of a signaling factor, or morphogen, that is secreted by an organizing center [1]. The ways in which morphogen gradients are established, particularly in vertebrates, remain obscure, although various suggestions have been made for the mechanisms by which signaling molecules traverse fields of cells. These include simple diffusion [2], "cytonemes" [3], filopodia [4], "argosomes" [5], and "transcytosis" [6]. In this study, we use a functional EGFP-tagged ligand to visualize long-range signaling in the *Xenopus* embryo in real time. Our results show that the TGF-β family member Xnr2 is secreted efficiently from embryonic cells, and a new method of tissue recombination allows us to investigate the way in which the morphogen traverses multiple cell diameters. This reveals that Xnr2 exerts long-range effects by diffusing rapidly through the extracellular milieu of nonexpressing cells. No evidence has been obtained for long-range signaling through cytonemes, filopodia, argosomes, or transcytosis. In demonstrating that long-range signaling in the early *Xenopus* embryo occurs by diffusion rather than by these alternative routes, our results suggest that different morphogens in different developmental contexts use different means of transport.**

## Results and Discussion

Formation of mesoderm and endoderm in the amphibian embryo occurs in response to graded inductive signals derived from the vegetal hemisphere of the embryo. These signals include transforming growth factor type β (TGF-β) superfamily members, including activin, Derrière, and Xnr1, 2, 4, 5, and 6 [7–13]. Although the concept of a morphogen gradient in the early vertebrate embryo is now generally accepted [14], it is not clear how such a gradient might be established. One possible mechanism is simple diffusion [2] (see Figure 1A), but more complex alternatives derive from work on *Drosophila* imaginal disks. These include cytonemes [3], filopodia [4], argosomal transport [5], and transcytosis [6] (see Figures 1B–1D).

Might such complex mechanisms operate during vertebrate development? Here, we study directly the movement of a member of the TGF-β family in the *Xenopus* embryo. TGF-β family members are produced as intracellular pro-proteins that subsequently dimerize and are cleaved to produce active secreted ligand. In previous work, tagged versions of the active form of the *Drosophila* TGF-β protein decapentaplegic were created by placing EGFP between the basic cleavage signal sequence and the mature region [6, 15]. In this study, we have created similar versions of the *Xenopus* TGF-β family members activin, Derrière, and nodal-related 2 (Xnr2). Additional constructs, in which HA tags were placed at the C termini of wild-type and EGFP-tagged proteins, were made (see Figure 2A).

### The Inducing Activities of Tagged Members of the TGF-β Family

To compare the inducing activities of tagged TGF-β family members with those of their parent molecules, we injected mRNAs encoding the tagged forms of activin, Derrière, and Xnr2 into *Xenopus* embryos at the 1 cell stage and observed their effects on development. In addition, real-time RT-PCR was carried out on isolated animal pole regions ("animal caps") to assess the abilities of the tagged proteins to activate the genes *Brachyury (Xbra)*, *Derrière (Der)*, and *Goosecoid (Gsc)*. In both assays, the EGFP-tagged forms of activin and Derrière proved to be significantly less active than the parent proteins (data not shown). However, the activities of EGFP-tagged versions of Xnr2 resemble those of the parent proteins. Thus, similar concentrations of Xnr2 and EGFP-Xnr2 are required both to activate the concentration-dependent expression of *Xbra*, *Derrière*, and *Gsc* in animal caps (Figure 2B) and to disrupt early development (Figures 2C–2E). Similarly, although the presence of a C-terminal HA tag reduces the specific activity of Xnr2, the inclusion of an EGFP moiety has no additional effect (data not shown).

The reduced activities of tagged versions of activin and Derrière may be explained by their three-dimensional structures. Although the monomeric structures of TGF-β family members are rather similar, their dimeric conformations vary significantly [16], and only some, including the nodal-related proteins [17], might permit the addition of an EGFP moiety to their N termini. Whatever the reason for the diminished activities of tagged activin and Derrière, we decided to focus on the long-range effects of Xnr2, whose activity is little affected by the inclusion of an N-terminal EGFP moiety.

### Tagged Versions of Xnr2 Are Correctly Processed and Secreted

Zygotic expression of *Xnr2* occurs in the vegetal hemisphere of the *Xenopus* embryo from the late blastula

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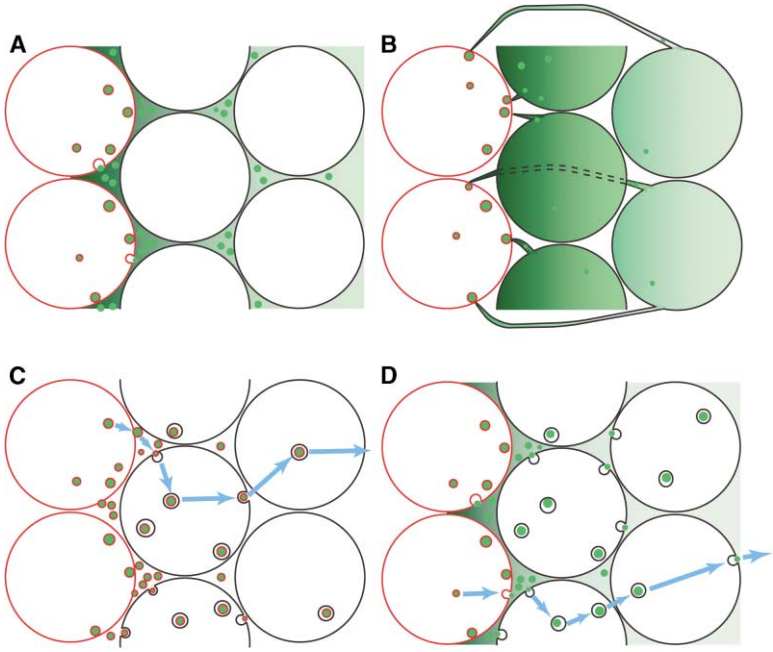


Figure 1. Mechanisms for Morphogen Gradient Formation

Red membranes represent expressing cells, black membranes represent receiving cells, and green shading represents ligand.
(A) Diffusion through extracellular space.
(B) Cytonemes project from distant to expressing cells and thereby produce an intracellular gradient of ligand [3]. An alternative mechanism involves the extension of projections from expressing to nonexpressing cells [4].
(C) Argosomal transport [5]. Membrane from expressing cells "chaperones" ligand through receiving tissue in "exovesicles."
(D) Transcytosis [6]. Ligand moves across receiving tissue by serial recycling through endocytosis and exocytosis.

stage [8], and RT-PCR allows the detection of low levels of maternal transcripts (P.H.W. and J.C.S., unpublished data). The early expression of Xnr2 and its potent inducing activity are consistent with the idea that it acts as a mesoderm-inducing factor, and indeed inhibition of all nodal-related signaling in the *Xenopus* embryo prevents mesoderm formation [18]. To confirm that tagged versions of Xnr2 are correctly processed and secreted in the *Xenopus* embryo, we analyzed embryo extracts and blastocoelic fluid from control and injected embryos by Western blotting (Figure 3A). Our results demonstrate that Xnr2-HA and EGFP-Xnr2-HA are correctly processed and secreted, and the presence of large amounts of processed ligand in the blastocoels of injected embryos, when it is compared with levels of the pro-protein, suggests that they are highly diffusible. Similar results were obtained when RNA injections were directed specifically to the vegetal hemisphere of the embryo, when most of the mature form of the Xnr2-HA protein is still found in the blastocoel, as judged by the protein's disappearance when the blastocoel roof is opened. The same is true of EGFP-Xnr2-HA, although more of this larger protein remains associated with the embryo (see Figure S1 in the Supplemental Data available with this article online).

Although substantial quantities of Xnr2 and EGFP-Xnr2 are secreted into the blastocoel, enough active material remains in the immediate extracellular milieu to disrupt normal development (Figures 2D and 2E). The secretion of nodal-related proteins into the blastocoel is unlikely to represent the route by which these proteins exert their inductive effect during normal development, for if this were so, the animal cap would be induced to form mesoderm. Indeed, there is evidence that such a route is precluded by the presence of an intrablastocoelic inhibitor of mesoderm induction [19].

## Long-Range Effects of Xnr2

To conform to the properties of a morphogen, a ligand must be able to induce target genes at long range. Previous work provides conflicting views about the long range signaling ability of Xnr2, with some groups asserting that the molecule acts essentially cell-autonomously and others concluding that it can act at a distance [12, 20–22]. In the present work, both Xnr2 and EGFP-Xnr2 proved to exhibit long-range activity. In one series of experiments, lineage-labeled animal caps expressing Xnr2, EGFP-Xnr2, or a constitutively active form of the type I activin receptor ALK4 [23] were juxtaposed with a control animal cap. The conjugates were cultured for 3 hr, fixed, and processed by in situ hybridization for expression of *Xbra* and by immunocytochemistry to visualize fluorescein lysine dextran (FLDx). In these experiments, Xnr2 and EGFP-Xnr2 proved to induce the target gene *Xbra* in nonexpressing tissue over a range of up to 12 cell diameters (Figure 3B–3D). Animal caps expressing constitutively active ALK4 did not activate *Xbra* expression in adjacent tissue (Figure 3E), arguing that the effects of Xnr2 and EGFP-Xnr2 are direct.

To address the possibility that wounding or other effects influence the long-range action of Xnr2 in these experiments, we created clones of Xnr2-expressing cells in the animal hemispheres of intact *Xenopus* embryos by injecting individual blastomeres at the 64 cell stage (Figures 3F–3I). As observed in the animal cap juxtaposition experiments, both Xnr2 and EGFP-Xnr2 exerted long-range effects, whereas constitutively active ALK4 behaved in a cell-autonomous manner.

We do not understand why the apparent signaling range of Xnr2 should vary in different experiments and experimental regimes; it may depend on the RNA expression vector or on the detailed timing of the experiments. This issue is under investigation.

## Visualizing Long-Range Signaling

To visualize the long-range movement of EGFP-Xnr2, we devised a method in which juxtaposed animal pole regions were allowed to adhere to a fibronectin-coated coverslip and thereby heal at one edge only (Figure 4A).

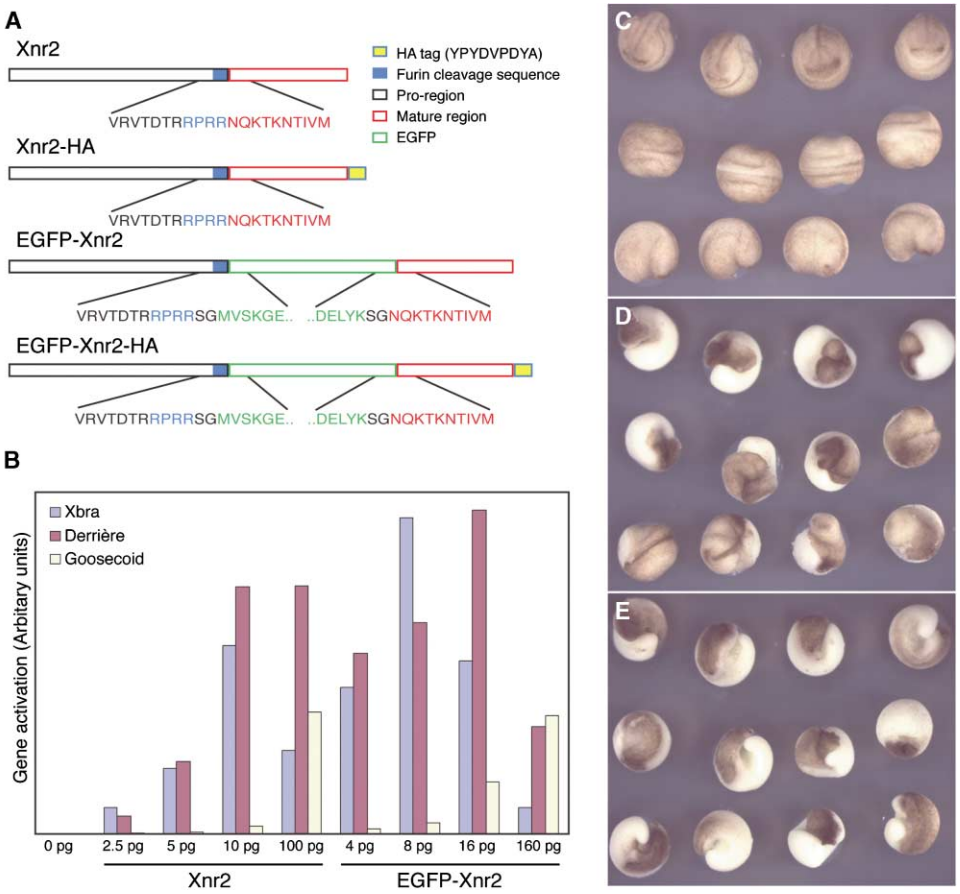


Figure 2. Tagged Xnr2 Constructs Retain Inducing Activity

(A) Schematic diagram showing tagged versions of Xnr2. EGFP was cloned between the endoprotease signal sequence (RPRR) and the mature ligand, and a single HA tag was added immediately before the stop codon of Xnr2.

(B) The specific activity of EGFP-Xnr2 resembles that of native Xnr2. *Xenopus* embryos were injected at the 1 cell stage with the indicated amounts of RNA and were then cultured to mid-blastula stage 8.5. Animal pole regions were isolated, cultured for a further 3 hr, and frozen. Expression of *Xbra*, *Derrière*, and *Goosecoid* was analyzed by real-time RT-PCR with the LightCycler (Roche), and figures were normalized to levels of ODC.

(C–E) Embryonic development is disrupted by similar concentrations of Xnr2 and EGFP-Xnr2. Embryos at the 1 cell stage were left uninjected (C) or were injected with 2.5 pg RNA encoding Xnr2 (D) or 4 pg RNA encoding EGFP-Xnr2 ([E]; the molar equivalent of 2.5 pg Xnr2). In both (D) and (E), ectodermal tissue is driven toward mesendodermal fates, leading to disruption of gastrulation.

Long-range signaling by EGFP-Xnr2 over a range of up to 10 cells was still observed in this experimental setup (Figure 4A′), and confocal microscopy revealed that tagged ligand does indeed pass through nonexpressing tissue (see below and Figures 4 and 5). To elucidate the mechanism by which Xnr2 exerts this long-range action, we decided to address each of the proposed mechanisms for gradient formation in turn.

*Xenopus* embryos are 1.2 mm in diameter, and our observations indicate that their blastomeres have diameters of 30–50 μm at the mid-blastula stage and 10–20 μm at the early gastrula stage. If cytonemes (Figure 1B) or other cellular extensions [4] were responsible for the long-range effects of Xnr2, these would need to be at least 100 μm in length. Although live *Xenopus* cells labeled with the CFP-GPI fluorescent membrane marker (see below) do extend protrusions, none were longer than 15 μm, and none extended more than 1 cell diameter (Figure 4B). These observations suggest that cytonemes or filopodia are not responsible for the long-range effects of Xnr2 in early *Xenopus* development.

Argosomes (Figure 1C) are membrane bound vesicles derived from ligand-producing cells; they are thought to transport inducing factors through responding tissue [5]. We investigated whether argosomes are responsible for the long-range effects of Xnr2 by coexpressing EGFP-Xnr2 with the fluorescent membrane marker CFP-GPI, which resembles the marker previously used by Greco and colleagues [5] to study argosomes in *Drosophila* imaginal disks. Animal caps derived from such embryos were juxtaposed with an uninjected animal cap (Figure 4C). In all specimens examined ($n > 10$), neither EGFP-Xnr2 nor EGFP-Xnr2-HA was ever observed in association with CFP-GPI-positive vesicles in the receiving animal caps. Although individual vesicles bounded by CFP-GPI were, on occasion, detected in the receiving caps (data not shown), these were never associated with EGFP-Xnr2. These observations do not support the

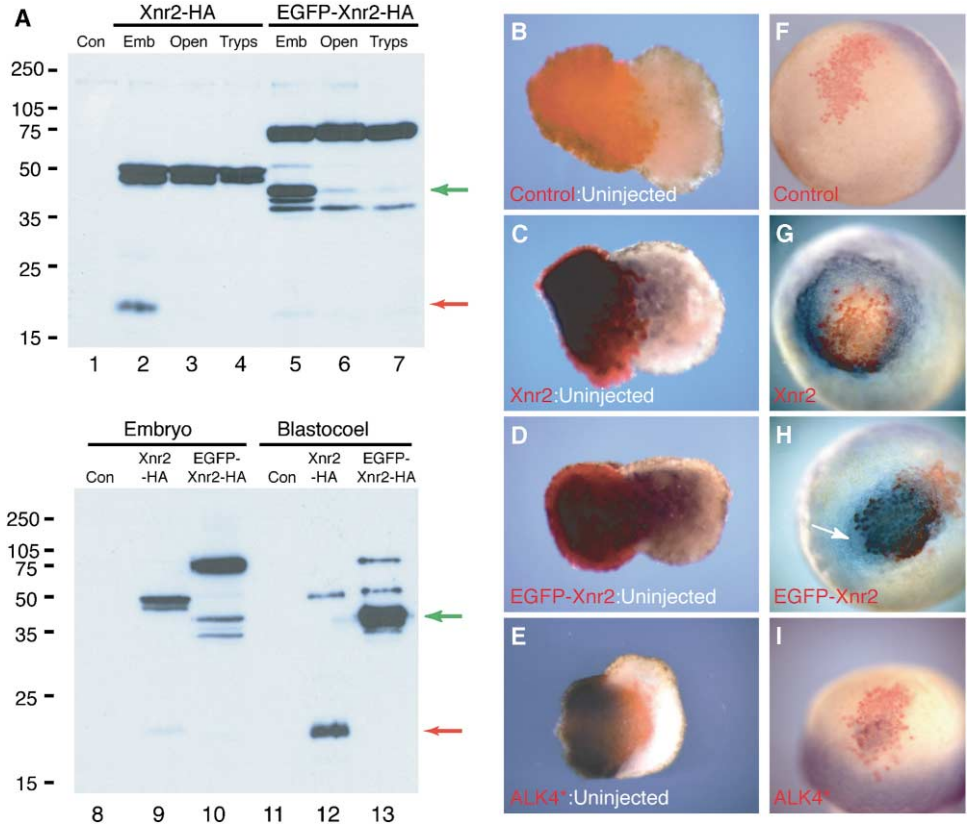


Figure 3. Xnr2 Is Secreted from Producing Cells and Activates Target Genes over a Long Range

(A) Embryos injected with RNA (200 pg) encoding the indicated tagged versions of Xnr2 were allowed to develop to the early gastrula stage, when they were treated as described below. Extracts were subjected to Western blotting with an anti-HA antibody. Slower-migrating bands represent unprocessed forms of the Xnr2 proteins. Green arrows indicate mature processed EGFP-Xnr2-HA, and red arrows indicate mature processed Xnr2-HA. (Lanes 1–7) Control embryos (lane 1) or injected embryos (lanes 2–7) were frozen immediately at early gastrula stage 10 (lanes 2 and 7), opened at the animal pole (without discarding any tissue) and cultured for 30 min before being frozen (lanes 3 and 6), or opened and cultured in 250 μg/ml porcine trypsin for 30 min before being frozen (lanes 4 and 7). Most secreted mature protein is lost after opening the embryos (lanes 3 and 6), and what remains is further diminished by trypsin treatment (lanes 4 and 7). (Lanes 8–13) To ensure that secreted mature ligand (lanes 3 and 6) was not degraded by proteases in the embryo culture medium, blastocoelic fluid was removed from ten intact injected embryos and subjected to Western blotting. Extracts of whole embryos (lanes 9 and 10; 0.5 embryo equivalent) show low levels of processed mature ligand. Secreted mature ligand is greatly enriched in blastocoelic fluid (lanes 12 and 13).

(B–E) Xnr2 and EGFP-Xnr2 activate *Xbra* at long range in animal cap conjugates. 100 ng FLDx (B), 100 ng FLDx + 10 pg Xnr2 (C), 100 ng FLDx + 16 pg EGFP-Xnr2 (D), or 100 ng FLDx + 300 pg constitutively active ALK4 (ALK4*) were injected into *Xenopus* embryos at the 1 cell stage. Animal caps were dissected and juxtaposed with uninjected animal caps. They were cultured for 3 hr, fixed, and processed by in situ hybridization for *Xbra* expression (blue) and by immunocytochemistry for FLDx (red). Upregulation of *Xbra* was never observed in control conjugates ([B]; n = 29), whereas both Xnr2 ([C]; 20/26) and EGFP-Xnr2 ([D]; 18/26) can upregulate *Xbra* in cells distant from the source. In a separate experiment, constitutively active ALK4 was found to act in a strict cell-autonomous fashion in all cases ([E]; n = 8). Xnr2 exerted long-range effects in five out of eight cases in this experiment, whereas EGFP-Xnr2 behaved cell-autonomously, perhaps because insufficient RNA was injected.

(F–I) Xnr2 and EGFP-Xnr2 activate *Xbra* at long range after injection of RNA into single blastomeres at the 64 cell stage of intact *Xenopus* embryos. 2 ng FLDx (F), 2 ng FLDx + 30 pg Xnr2 (C), 2 ng FLDx + 40 pg EGFP-Xnr2 (D), or 2 ng FLDx + 30 pg constitutively active ALK4 (ALK4*) were injected into single blastomeres of *Xenopus* embryos at the 64 cell stage. Embryos were allowed to develop to early gastrula stage 10.5 and were then processed by in situ hybridization for *Xbra* expression (blue) and by immunocytochemistry for FLDx (red). Upregulation of *Xbra* was never observed in control embryos ([F]; n = 14), whereas both Xnr2 ([G]; 14/22) and EGFP-Xnr2 ([H], arrow; 7/16) cause upregulation of *Xbra* in cells distant from the source. Circles of *Xbra* expression, as shown in (G), were observed only once in response to EGFP-Xnr2 (H), suggesting that this tagged molecule has a slightly more restricted range than the parent protein. Constitutively active ALK4 was unable to activate nonautonomous expression of *Xbra* ([I]; n = 16, of which 13 showed exogenous *Xbra* expression).

notion that argosomes transport Xnr2 from expressing to nonexpressing tissue.

Preliminary results suggested that transcytosis (Figure 1D) plays no role in the induction or patterning of *Xenopus* mesoderm. Rab5 is a small GTPase that controls vesicle transport from the plasma membrane to the early endosome [24]. Injection of RNA encoding constitutively active or dominant-negative *Drosophila* Rab5 [6] into the equatorial region of *Xenopus* embryos at the 4 cell stage had no effect on the expression domain or level of activation of endogenous *Xbra*, even though the constitutively active form of Rab5 caused the formation of large, pigment-filled early endosomes (data not shown). This observation is of interest because endocy-

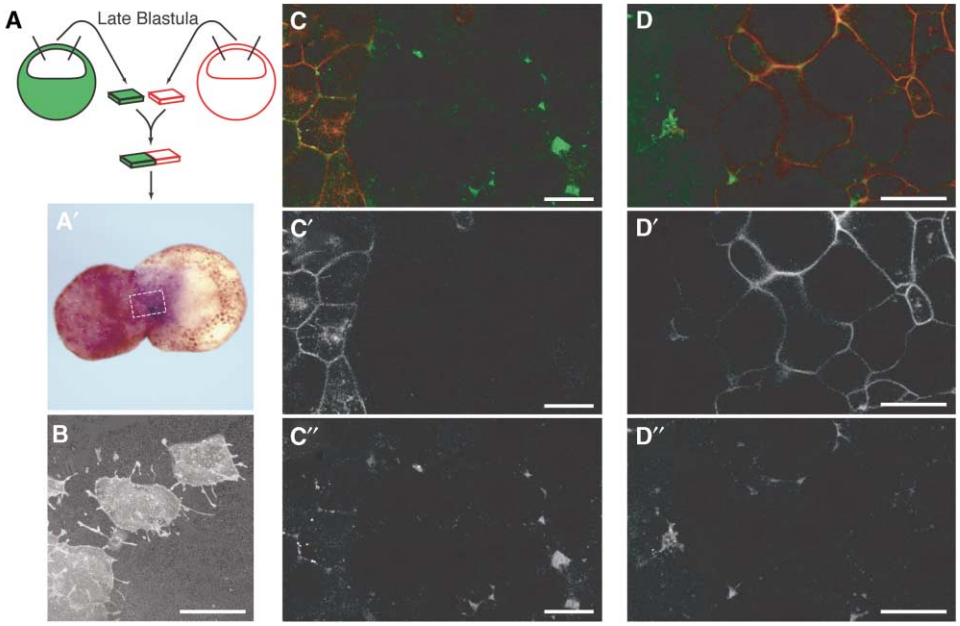


Figure 4. Neither Argosomal Transport Nor Transcytosis Is Responsible for the Long-Range Effects of EGFP-Xnr2

(A) Schematic diagram showing two animal caps juxtaposed to allow observation of tagged proteins in real time. Animal caps from embryos injected with the desired RNAs are dissected at the late blastula stage and juxtaposed on a fibronectin-coated coverslip.

(A′) Long-range signaling occurs in this experimental setup as it does in the experiments described in Figure 3. Animal caps derived from embryos injected with 400 pg EGFP-Xnr2 and 2 ng FLDx were juxtaposed as described in (A) and cultured for 4 hr before being processed by in situ hybridization for *Xbra* expression (blue) and by immunocytochemistry for FLDx (red). Long-range upregulation of *Xbra* was observed in all 15 conjugates examined. No *Xbra* expression was observed in control conjugates lacking EGFP-Xnr2 ($n = 14$; data not shown), and long-range signaling was not observed in experiments in which constitutively active ALK4 replaced EGFP-Xnr2, although in one case (out of 14), *Xbra* was activated in cells immediately adjacent to the FLDx-labeled tissue (data not shown). In the images that follow, the passage of tagged ligand from one animal cap to another is followed by confocal microscopy. With the exception of (B) below, all images represent optical sections taken at the level of the nucleus of the cell adjacent to the coverslip. The field of view in (C)–(C″) and (D)–(D″) resembles that outlined in (A′).

(B) Z series projection of cells labeled with the membrane marker CFP-GPI. Blastomeres extend projections into their immediate environment, but none extend further than a single cell diameter. The scale bar represents 20 μm.

(C–C″) EGFP-Xnr2 does not exert long-range effects with argosomes. RNA (400 pg) encoding EGFP-Xnr2 (C″) was coinjected into *Xenopus* embryos with RNA (500 pg) encoding the membrane marker CFP-GPI (C′). Animal caps from such embryos were juxtaposed with uninjected caps. EGFP-Xnr2 in receiving caps was not surrounded by CFP-GPI-labeled membrane but is present in abundance in the extracellular space. (C) is a merged image of (C′) and (C″). The scale bars represent 20 μm.

(D–D″) EGFP-Xnr2 does not exert long-range effects by transcytosis. Animal caps expressing EGFP-Xnr2 (D″) were juxtaposed with caps expressing CFP-GPI (D′). EGFP-Xnr2 in the receiving cap is extracellular and is not detected in CFP-GPI-bounded cells or vesicles. (D) is a merged image of (D′) and (D″). The scale bars represent 20 μm. Amounts of injected RNA are as described in (C)–(C″).

tosis has been implicated in TGF-β signaling in other systems [25], and its role in early *Xenopus* development deserves further study.

We next investigated in detail whether transcytosis plays a role in the transport of EGFP-Xnr2; we juxtaposed animal caps expressing tagged ligand with caps expressing CFP-GPI. If ligand were being passed from cell to cell via the endocytic machinery, much of the EGFP-Xnr2 within receiving tissue would be found within cells bounded by the CFP-GPI marker. We note that ligand might not be associated with CFP-GPI within recipient cells because this marker associates with lipid-rich domains involved in the degradative pathway [26].

In all conjugates examined ($n > 10$), the bulk of the EGFP-Xnr2 in the receiving cap was extracellular and confined to the interstices between cells (Figure 4D), more consistent with the idea that ligand traverses between cells by diffusion. This localization of EGFP-tagged ligand contrasts markedly with that observed in studies with *Drosophila* wing disks [3, 5, 6]. Small amounts of intracellular fluorescence in receiving cells were observed, and it is possible that this represents receptor bound ligand that is internalized in the course of signaling. It is also possible that degradation of endocytosed ligand acts as a mechanism for controlling the slope of a gradient of Xnr2. Indeed, differential degradation in adjacent compartments in the *Drosophila* embryo causes Wingless signaling to occur in an asymmetric manner [27].

We note that our inability to observe argosomes or transcytotic vesicles is unlikely to be due to the limited depth (three-quarters of a cell) to which the confocal microscope can penetrate yolky *Xenopus* tissue. When albino animal caps are viewed from the external surface instead of from the blastocoelic side, we are still unable to detect argosomes or transcytotic vesicles, although there is significantly less extracellular EGFP-Xnr2 surrounding these much more closely packed cells.

Together, these results suggest that EGFP-Xnr2 exerts long-range effects in *Xenopus* tissue by simple dif-

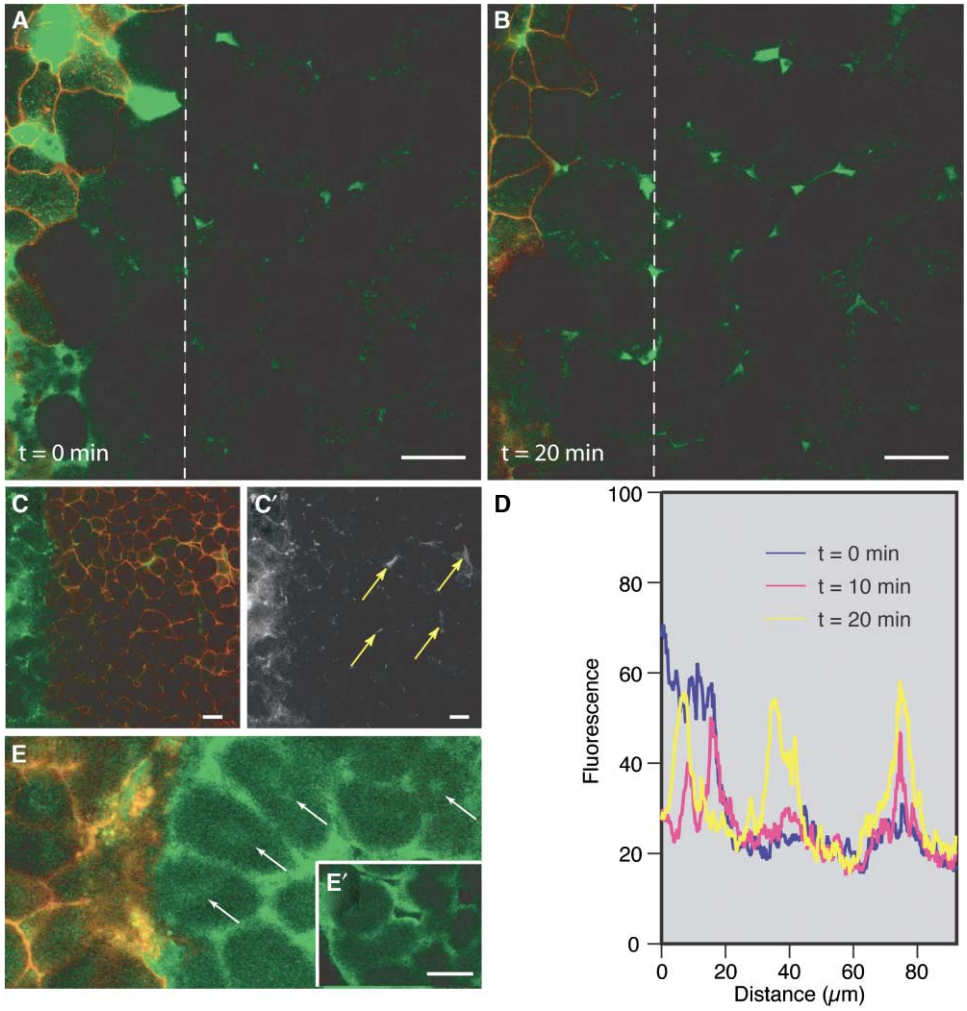


Figure 5. EGFP-Xnr2 Exerts Long-Range Effects by Diffusion and Thereby Activates the Smad Signal Transduction Pathway

(A and B) Two images of the same animal cap conjugate; images were taken at an interval of 20 min and therefore represent frame 1 and frame 40 of a time course (see Movie 1). An animal cap expressing EGFP-Xnr2 (400 pg RNA) and CFP-GPI (500 pg RNA) (to the left of the image) was juxtaposed with a control animal cap (to the right). EGFP-Xnr2 in the receiving tissue is almost exclusively extracellular and has traversed 2–3 cell diameters over the course of the experiment (see Figure S2). Tissue to the right of the dotted line was subjected to a quantitative analysis of fluorescence intensity (see [D]).

(C and C′) Within 2 hr of animal cap juxtaposition, EGFP-Xnr2 extends at least 200 μm (about 11 cell diameters) into receiving tissue. An animal cap expressing EGFP-Xnr2 (to the left of the images) was juxtaposed with a cap expressing CFP-GPI (to the right). (C) shows a merged view of the GFP and CFP channels. (C′) shows the GFP channel. Note the accumulation of extracellular fluorescence at distances up to 200 μm from expressing cells (yellow arrows). The scale bars represent 20 μm. Amounts of injected RNA are as described in (A) and (B).

(D) Quantitation of fluorescence intensity in the receiving animal cap shown in (A) and (B). The LineProfile function of the LaserSharp 2000 Software (BioRad) was used to measure fluorescence intensity within the indicated portion (to the right of the dotted line) of the receiving animal cap 60 min after initial juxtaposition (t = 0 min; blue line) and 10 and 20 min thereafter (maroon and yellow lines, respectively). Note that total fluorescence in the receiving caps (measured in arbitrary units) increases over time and that, consistently with the images in (A) and (B), the distribution of fluorescence is patchy, with accumulations corresponding to extracellular aggregates.

(E and E′) EGFP-Xnr2 activates intracellular signal transduction pathways. Animal caps expressing GFP-Smad2 (500 pg RNA) were juxtaposed with caps expressing EGFP-Xnr2 (400 pg RNA) and CFP-GPI (500 pg RNA) (E) or were cultured alone (E′). Receptor activation occurring through the long-range effects of EGFP-Xnr2 causes GFP-Smad2 to translocate to the nucleus (arrows in [E]). No such translocation is observed in control caps injected with GFP-Smad2 alone (E′). The scale bars represent 20 μm.

fusion. To follow this process in real time and to address the rate of diffusion, we made time-lapse movies. Our results indeed show that movement of EGFP-Xnr2 occurs by diffusion through the extracellular space, and they indicate that ligand can travel 30–40 μm within 20 min (Figures 5A and 5B; Figure S2; Movie 1) and at least 200 μm, or approximately 10 cells, within 2 hr of animal cap juxtaposition (Figures 5C and 5C′). This rate of diffusion is similar to the inferred diffusion rates of activin in animal pole tissue [28] and that of Nodal in the chick embryo [17]. It has proved difficult to study the distribution of EGFP-Xnr2 in receiving animal caps in a quantitative manner because it accumulates in interstices between cells, perhaps in association with the extracellular

matrix. This nonuniform distribution is confirmed by analysis of EGFP fluorescence in the juxtaposed animal cap with the LineProfile function of the LaserSharp 2000 Software (BioRad) (Figure 5D), although the analysis does demonstrate clearly that levels of fluorescence in the receiving cap increase over time.

To confirm the biological activity of EGFP-Xnr2 in such experiments, we juxtaposed animal caps expressing both EGFP-Xnr2 and CFP-GPI with caps expressing GFP-Smad2 [29]. Smad2, an intracellular transducer of activin-like signaling, is phosphorylated and translocated to the nucleus in response to ligand-receptor interaction. One hour after conjugation, previously cytoplasmic GFP-Smad2 is translocated to the nucleus in response to the EGFP-Xnr2 (Figures 5E and 5E′).

## Conclusions

Our results indicate that *Xenopus* Xnr2 acts on distant cells by diffusing through extracellular space. We have obtained no evidence for the existence of cytonemes, and neither argosomal transport nor transcytosis appears to play a role. The formation of a gradient of Xnr2 and perhaps other nodal-related proteins is likely to play an important role in mesodermal patterning during *Xenopus* development [18], although we note that nodal activity is also regulated by extracellular factors such as antivin/lefty [30]. The establishment of mesodermal pattern in *Xenopus* will involve closely regulated interactions between nodal-related proteins and their inhibitors, and our results suggest that many of these interactions will involve long-range movement of nodal-related proteins in the extracellular space.

### Experimental Procedures

#### Embryos and In Situ Hybridization

Embryos of *Xenopus laevis* were obtained, staged, cultured, microinjected, and subjected to in situ hybridization as described [12]. Visualization of FLDx was as described [31]. Western blotting of embryos and blastocoelic fluid used peroxidase-conjugated rat monoclonal antibody (clone 3F10, Roche Diangostics) to detect the HA epitope.

#### RT-PCR

Real-time PCR with the LightCycler (Roche) was carried out with the manufacturer's RNA Amplification Kit. All determinations included a negative control, and a serial dilution of embryo RNA was used to create a standard curve. Primers specific for *Derrière*, *Xbra*, and *ornithine decarboxylase* (*ODC*) were as described [10, 32, 33]. *Gsc* primers were (upstream) 5′-TGGCAAGGAGGGTTCATCTCAGAG-3′ and (downstream) 5′-ATCCAGCTATCCCAATGTGCAACT-3′. All values were normalized to the level of ODC in each sample.

#### Injection Constructs

EGFP- and HA-tagged constructs (Figure 2A) were made by PCR with standard techniques. The open reading frames (ORFs) of all constructs were cloned into pCS2+. Where indicated, single copies of the HA epitope were inserted immediately before the stop codon of each ORF.

#### Confocal Microscopy

Confocal imaging was carried out with a BioRad Radiance 2000/Nikon Eclipse E800 microscope. The confocal apparatus was controlled with LaserSharp 2000 software (BioRad). Manipulation of confocal images and time-lapse movies used the software packages Imaris, Adobe Premiere 6.0, and Apple QuickTime. The fluorescence quantitation shown in Figure 5D used the LineProfile function of the LaserSharp 2000 software.

#### Supplemental Data

Supplemental Figures and a Supplemental Movie are available online at http://www.current-biology.com/cgi/content/full/14/21/1916/DC1.

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