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Original Article

Xantivin suppresses the activity of EGF-CFC genes to regulate nodal signaling

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ABSTRACT ***Lefty, antivin*** **and related genes act in a feedback inhibition mechanism for nodal signaling at a number of stages of vertebrate embryogenesis. To analyze the function of the feedback inhibitor of nodal signaling, Xantivin in *Xenopus* embryos, we designed a morpholino antisense oligonucleotide (XatvMO) for this gene. XatvMO caused the expansion of mesodermal tissue and head defects. XatvMO-injected gastrulae showed up-regulated expression of the mesodermal markers *Xbra, Xwnt8, Xnot,* and *Chordin,* suggesting expansion of the trunk-tail organizer. As expected, depletion of *Xantivin* also up-regulated nodal signaling as confirmed by the enhanced ectopic expression of *Xantivin* mRNA, a known target gene of nodal signaling. Furthermore, we investigated the relationship between *Xantivin* and the EGF-CFC gene *FRL-1,* which is a component of the nodal receptor. In animal cap assays, *FRL-1* could not induce expression of nodal-responsive genes, but could up-regulate expression of these genes when *FRL-1* was coinjected with a low dose of *Xnr1;* coinjection of *Xantivin* suppressed this up-regulation by *FRL-1*. We also found that *Xantivin* can rescue the caudalized phenotype induced by overexpression of *FRL-1*. Co-immunoprecipitation assays showed that *Xantivin* interacted with the EGF-CFC proteins, FRL-1 and cripto. Taken together, these results suggest that *Xantivin* opposes the activity of EGF-CFC genes and thereby antagonizes nodal signaling.**

KEY WORDS: *nodal, antivin, lefty, EGF-CFC gene, Xenopus laevis*

Introduction

Mesoderm induction is the first inductive event in vertebrate development. Several members of the TGF-β superfamily have been identified in *Xenopus* as mesoderm-inducing factors, including the *nodal*-related genes, *Xnr1*, *Xnr2*, *Xnr4*, *Xnr5,* and *Xnr6* (Jones *et al.,* 1995, Joseph and Melton 1997, Takahashi *et al.,* 2000) *Vg1* (Weeks and Melton 1987), *derrière* (Sun *et al.,* 1999), and activin (Asashima *et al.,* 1990). Nodal signaling is a crucial developmental event in a number of vertebrate species. Mice deficient in *nodal*, the founding member of this subfamily of genes, show no evidence of mesoderm induction(Conlon *et al.,* 1994). In *Xenopus*, mesoderm induction can be inhibited by a nodal antagonist, *Cerberus-short form* (Piccolo *et al.,* 1999), and by a dominant-negative *nodal* construct (Onuma *et al.,* 2002, Osada and Wright 1999). In zebrafish, double mutants of the *nodal*-related genes *cyclops* and *squint* lack mesendoderm (Feldman *et al.,* 1998). Both *nodal*-related gene products and activin activate ActRI and ActRII (Hill 2001). Recent evidence suggests that a number of TGF-β signals, including nodal, Vg1, and GDF1, require EGF-CFC coreceptors (Cheng *et al.,* 2003, Gritsman *et al.,* 1999, Schier and Shen 2000, Whitman 2001). These proteins have a variant epidermal growth factor (EGF)-like domain and a cripto-FRL-1-cryptic (CFC) domain. They have been cloned from several vertebrate species and include mouse *cripto*, *Xenopus FRL-1,* and zebrafish *one-eyed pinhead* (Shen and Schier 2000). Mouse Cripto was shown to bind to the ActRI, Alk4, leading to the phosphorylation of the intracellular mediator Smad2 with nodal and the activation of downstream signaling (Bianco *et al.,* 2002, Yeo and Whitman 2001).

Abbreviations used in this paper: ActRI, activin receptor type I; ActRII, activin receptor type II; β-gal, β-galactosidase; EGF-CFC, epidermal growth factor-like-Cripto-FRL-1-Cryptic; HA, hemagglutinin tag; MO, morpholino antisense oligonucleotide; StdMO, standard MO; TGF-β, transforming growth factor β; Xatv, Xantivin.

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Nodal signaling has also been implicated in several other developmental processes such as anterior-posterior patterning (Schier and Shen 2000). In zebrafish, overexpression of *antivin*, an inhibitor of nodal signaling, depletes posterior fates (Thisse *et al.,* 2000). In *Xenopus*, an inhibitor of nodal signaling, *Cerberus-short form*, induces head structure formation in the ventral marginal zone, and overexpression of *Xnr1* after the gastrula stage causes a head structure defect (Piccolo *et al.,* 1999). These results suggest that nodal signaling acts as a posteriorizing signal. Some members of the EGF-CFC family are also involved in anterior-posterior patterning. *Cripto*-deficient mice lack posterior structures (Ding *et al.,* 1998) while overexpression of *FRL-1* causes posteriorization (Kinoshita *et al.,* 1995, Yokota *et al.,* 2003). Therefore nodal signaling via EGF-CFC genes is likely to be important in posterior development.

The signal molecules *Lefty*, *antivin,* and related genes are regulated by nodal signaling. These genes have been isolated from zebrafish (Bisgrove *et al.,* 1999, Thisse and Thisse 1999), *Xenopus*, (Branford *et al.,* 2000, Cheng *et al.,* 2000, Tanegashima *et al.,* 2000), chicks (Ishimaru *et al.,* 2000, Rodriguez Esteban *et al.,* 1999), mice (Meno *et al.,* 1997, Meno *et al.,* 1996), and humans (Kosaki *et al.,* 1999), and their function and transcriptional regulation are highly conserved. In addition, the promoter of the mouse *lefty* gene has a binding site for FAST2, a mediator of activin/nodal signaling, which forms a complex with activated Smad2 (Saijoh *et al.,* 2000). The function of the *lefty/antivin* family was first analyzed with respect to left-right axis formation using a *lefty-1*-deficient mouse (Meno *et al.,* 1998). In zebrafish and *Xenopus* embryos, overexpression of *lefty* or *antivin* inhibits mesoderm induction and antagonizes the activity of *activin* and *nodal*-related genes (Bisgrove *et al.,* 1999, Cheng *et al.,* 2000, Tanegashima *et al.,* 2000, Thisse and Thisse 1999). *Lefty-2*-knockout mice exhibit excessive mesoderm formation and an expanded primitive streak, an opposite phenotype to that of *nodal*-knockout mice (Conlon *et al.,* 1994, Meno *et al.,* 1999, Zhou *et al.,* 1993). These results suggest that the members of the lefty/antivin subfamily act as feedback inhibitors of nodal signaling to regulate mesoderm induction. Although *lefty/antivin* has been well characterized at the transcriptional and functional levels, little is known about the biochemical interactions of *lefty/antivin*. Two lines of evidence suggest that *lefty/antivin* may competitively bind to ActRII to inhibit nodal signaling. First, overexpression of the ActRII or its extracellular domain suppressed the phenotype caused by overexpression of *antivin* (Meno *et al.,* 1999, Thisse and Thisse 1999). Second, the expansion of mesoderm in the *lefty2* mutants is suppressed by a deficiency in *actRIIB* (Sakuma *et al.,* 2002). However, it has not been determined whether lefty/antivin interacts directly with ActRIIs or with another receptor that acts in nodal signaling.

To analyze the function of the lefty/antivin-related *Xantivin* gene in *Xenopus* embryos, we designed a morpholino antisense oligonucleotide (MO) against *Xantivin* (XatvMO). XatvMO-injected gastrulae had up-regulated expression of mesodermal markers, suggesting expansion of the trunk-tail organizer. The XatvMO-injected phenotype resembled that of embryos injected with *FRL-1* reported previously (Kinoshita *et al.,* 1995, Yokota *et al.,* 2003), suggesting that *Xatv* can suppress the activity of an EGF-CFC gene. *FRL-1* up-regulated the expression of nodal

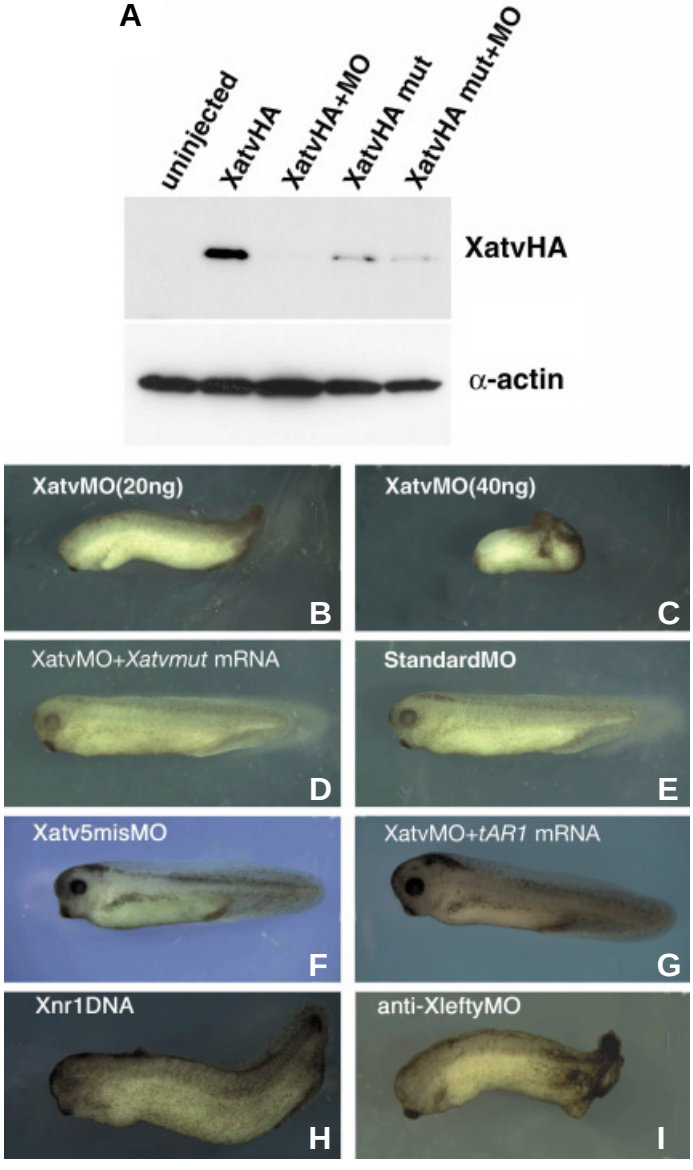

Fig. 1. *XatvMO* caused posteriorizing phenotypes. (A) XatvMO *inhibited the translation of* Xatv *and this inhibition was dependent on the* XatvMO *target sequence. Xatv protein which has a HA-epitope tag, was detected using an anti-HA antibody.* α*-Actin served as the loading control.* XatvHA *mRNA (*Xatv *with a* HA *epitope) and* XatvHAmut *mRNA (Xatv with no* XatvMO *target sequence) were injected with or without coinjection of* XatvMO *into oocytes. Oocyte lysates with uninjected controls were used for Western blotting.* XatvMO *inhibited the translation of* XatvHA*, but not of* XatvHAmut *mRNA. (B-F, I)* XatvMO, StdMO, Xatv5misMO, anti-XleftyMO, *or* XatvMO *was vegetally injected into each blastomere of 4-cell stage embryos, which were grown to tadpole stage.* **(B)** *Embryo injected with 20 ng* XatvMO. **(C)** *Embryo injected with 40 ng* XatvMO. **(D)** *Embryo injected with 40 ng* XatvMO *rescued by 2 pg* Xatvmut *mRNA. (E,F) Embryos injected with 40 ng StdMo* **(E)** *or Xatv5misMO* **(F)** *showed no developmental abnormality.* **(G)** *Embryo injected with 40 ng* XatvMO *rescued by 100 pg* tAR1 *mRNA.* **(H)** pCS2-Xnr1 *DNA (50 pg) was injected into dorsal vegetal blastomeres of 8-cell stage embryos, which showed a similar phenotype to the* XatvMO*-injected embryo.* **(I)** *Embryos injected with 2.5 ng anti-*XleftyMO *showed a caudalized phenotype.*

pathway target genes, whereas coinjection of *Xatv* suppressed them in the animal cap. Furthermore, co-immunoprecipitation assays showed that *Xatv* interacts with the EGF-CFC proteins, FRL-1 and cripto. These results suggest that *Xatv* suppresses the activity of EGF-CFC genes to regulate nodal signaling and anterior-posterior axis formation.

Results

Depletion of Xatv *causes up-regulation of nodal signaling*

Morpholino oligonucleotides (MOs), which inhibit the translation of target genes, are useful tools for the analysis of gene function in *Xenopus* (Heasman 2002). To examine the function of the *Xenopus lefty/antivin*-related gene *Xatv*, we designed a specific MO (XatvMO) and tested its activity using the *Xenopus* oocyte expression system (Fig. 1A). On Western blot the *XatvHA* protein, which consists of Xatv tagged with the HA epitope at the C-terminus, was approximately 35 kDa, corresponding to the processed form of Xatv (Fig. 1A, lane 2). We did not detect the unprocessed form of *XatvHA* protein (42 kDa) in this condition (10ng of *XatvHA* or *XatvHA* mutant mRNA into an oocyte) but detect it when injecting 40ng of *XatvHA* mRNA (data not shown). The co-injection of *XatvHA* mRNA and XatvMO showed that XatvMO specifically inhibited the translation of *XatvHA* (Fig. 1A, lane 3). *XatvHA* mutant (*XatvHAmut*) did not contain the target XatvMO sequence and produce less amount of protein than wild type mRNA (Fig. 1A, lane 4). This reduced translational efficiency might be occurred by nucleotide substitution of *XatvHAmut* mRNA to wild type mRNA. However, the level of the translated *XatvHA* protein was comparable to that of XatvMO coinjected one when we used *XatvHAmut* mRNA (Fig. 1A, lanes 4, 5). Embryos injected with 20 ng XatvMO showed anterior structure defects and a twisted axis and tail (Fig. 1B, Table 1). More extensive defects were apparent with higher doses (40 ng) of XatvMO (Fig. 1C, Table 1). The higher-dose phenotypes could be rescued by coinjection of 2 pg *Xatvmut* mRNA (Fig. 1D, Table 1), suggesting that the XatvMO-induced phenotypes were specifically induced by the depletion of *Xatv* protein. In contrast, 40 ng StdMO or Xatv5misMO had no apparent effects on development, supporting the specific action of XatvMO (Fig. 1 E,F).

Previous results suggest that lefty/antivin may block ActRII and antagonize nodal signaling (Meno *et al.,* 1999, Sakuma *et al.,*

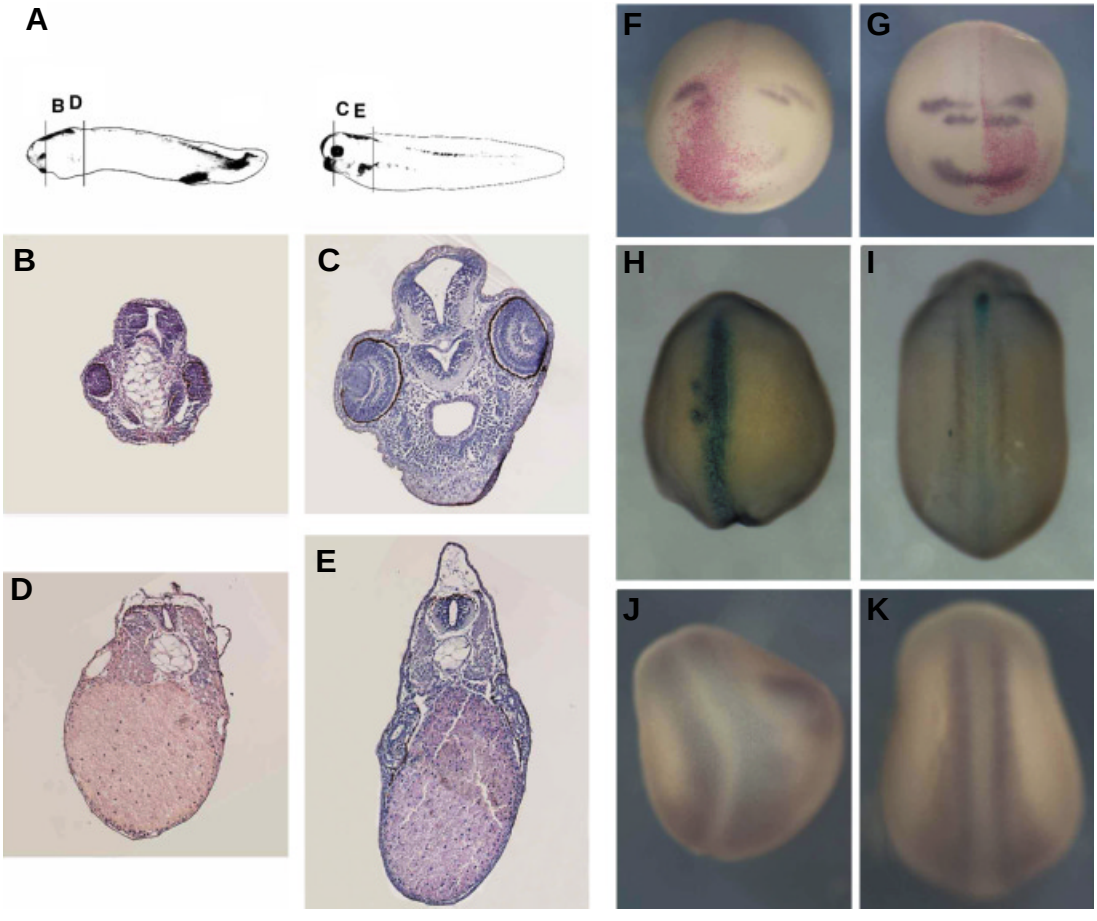

Fig. 2. *XatvMO*-injected embryos have enlarged mesodermal tissues. (A) *Diagrams of embryos injected with* StdMO *and* XatvMO. *Lines indicate the approximate position of sections shown in (B-E).* XatvMO *(20 ng) or* StdMO *(40 ng) was injected into the vegetal region of 4-cell stage embryos.* **(B)** *Section of the head region of an embryo injected with* XatvMO *shows that forebrain and eye structures were reduced, and an expanded notochord had invaded anteriorly.* **(C)** *Section of the head region of an embryo injected with* StdMO. **(D)** *Section of the posterior region of an embryo injected with* XatvMO *shows an expanded notochord and muscle.* **(E)** *Section of the posterior region of an embryo injected with* StdMO. *(F,G) Injection of* XatvMO *caudalized the neuroectoderm.* XatvMO *(25 ng) or* StdMO *(25 ng) was coinjected with 250 pg* β-gal mRNA *into one blastomere of a 4-cell stage embryo, which was grown to early neurula to detect* BF-1 *(forebrain) and* Krox20 *(hindbrain) expression.* **(F)** *Embryos injected with XatvMO lost the expression of* BF-1 *but not of* Krox20. **(G)** *An embryo injected with StdMO marked with blue in the future forebrain and hindbrain, which was marked by the expression of* BF-1 *and* Krox20, *respectively.* (H-K) *XatvMO or StdMO was vegetally injected into each blastomere of a 4-cell stage embryo, which was grown to late neurula.* **(H)** *Immunostaining with monoclonal antibody Tor70, revealed that embryos injected with* XatvMO *had enlarged notochords.* **(I)** *Normal notochord of an embryo injected with* StdMO *specifically stained by Tor70.* **(J)** *The expanded expression of* XmyoD *was detected in an embryo injected with* XatvMO. **(K)** *The expression of* XmyoD *was detected in the somite of an embryo injected with* StdMO.

2002, Thisse and Thisse 1999). We found that the posteriorizing phenotype caused by XatvMO was rescued by the coinjection of *tAR1* (Fig. 1G), a dominant-negative form of *Xenopus* ActRII (Hemmati-Brivanlou and Melton 1992). Embryos injected with pCS2-*Xnr1*DNA showed anterior defects and a twisted axis and tail, consistent with a previous report (Piccolo *et al.,* 1999), resembling the phenotype of XatvMO-injected embryos (Fig. 1H; n=35, 71%). Taken together, these findings indicate that depletion of *Xatv* results in a caudalized phenotype, which may be the result of an up-regulation of nodal signaling.

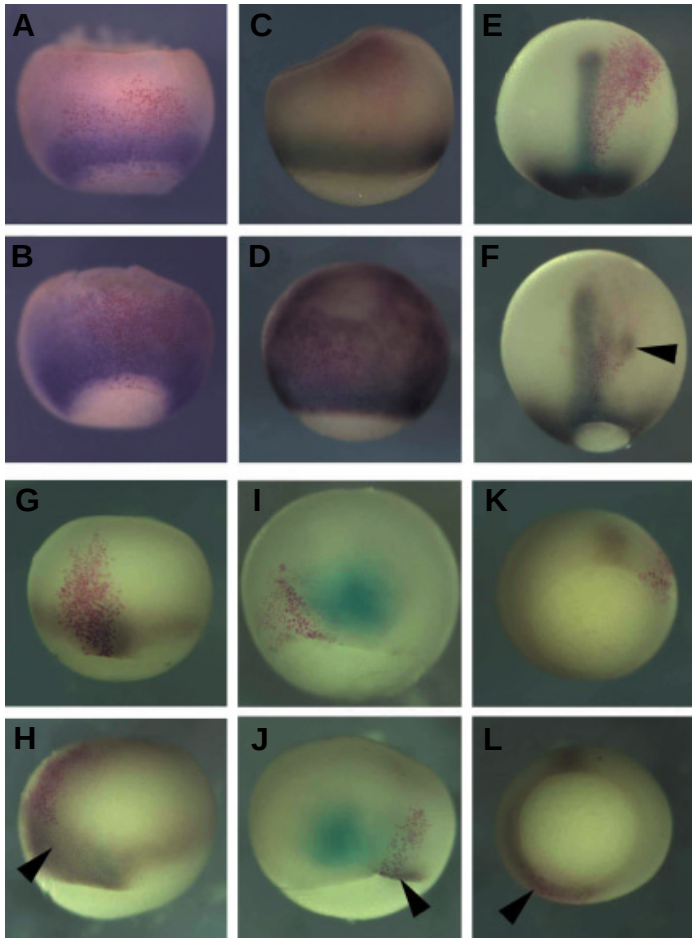

Fig. 3. *XatvMO* disturbs the expression of mesodermal markers. *(A-L) 25 ng* StdMO *(A,C,E,G,I,K) or 25 ng* XatvMO *(B,D,F,H,J,L) were coinjected with 250 pg* β-gal mRNA *into* (E-L) *one or* (A-D) *two blastomeres of 4-cell stage embryos. The injected regions were visualized by red, indicating β-galactosidase activity. Injected embryos were grown to mid to late gastrulae for whole-mount* in situ *hybridization; signal is indicated by blue staining. Arrowheads indicate the* XatvMO*-injected region.* **(A,B)** *The expression of* Xwnt8 *(ventral view) and* **(C,D)** Xbra *(lateral view) was detected in the marginal zone, which was the future mesoderm of embryos injected with* StdMO*. However, the injection of* XatvMO *induced a dispersed expression into the animal region. (E-J) Dorsal view of late gastrulae (* Xbra*) and mid gastrulae (* Xnot, Chordin*).* **(E)** *Expression of* Xbra, **(G)** Xnot *and* **(I)** Chordin *was detected in the axial mesoderm of embryos injected with* StdMO *and was up-regulated in the* XatvMO*-injected region.* **(F)** Xbra; **(H)** Xnot *and* **(J)** Chordin. *(K,L) Vegetal view of mid gastrulae. The ectopic expression of* Xatv *mRNA was detected in the* XatvMO*-injected region* **(L)**, *but not in the StdMO-injected region* **(K)** .

Recently another MO against a *lefty*/*antivin*-related gene in *Xenopus* (anti-XleftyMO) has been reported to cause exo-gastrulation (Branford and Yost 2002), indicating that *lefty*/*antivin*-related genes in *Xenopus* are required for normal gastrulation. To reconcile these different phenotypes, we injected various amounts of the anti-XleftyMO into *Xenopus* embryos. At high doses (40 ng), this MO caused exo-gastrulation (data not shown) as reported (Branford and Yost 2002). However, at low doses (2.5 ng) the phenotypes produced by the XleftyMO and the XatvMO were similar (Fig. 1I, Table 1).

XatvMO-*injected embryos have expanded caudal mesoderm*

Histology showed that embryos injected with 20 ng XatvMO had reduced forebrain and eye structures, confirming that XatvMO caused defects to anterior structures (Fig. 2B). In addition, an expanded notochord invaded into the anterior region where no notochord existed in the normal embryo (Fig. 2 B,C). In the posterior region, embryos injected with XatvMO had enlarged notochord and muscle (Fig. 2 D,E). Whole-mount *in situ* hybridization using the forebrain marker, *BF-1,* and the hindbrain marker, *Krox20*, was used to assess the effects of Xatv ablation in more detail. XatvMO or StdMO was coinjected with *β-gal* mRNA into one blastomere of a 4-cell stage embryo. *BF-1* transcripts were detected in tissue fated to be forebrain in the embryos injected with StdMO (Bourguignon *et al.,* 1998) (Fig. 2G), but were not detected in the region where XatvMO

TABLE 1

MO-INJECTED PHENOTYPES AND THEIR RESCUED EMBRYOS

			Anterior defect		
		Number	Anterior defect	Severe anterior defect with tail-like protrusion	Normal
XatvMO	20 ng	45	62%	31%	7%
XatvMO	40 ng	92	36%	64%	0%
XatvMO+Xatvmut	40 ng+2 pg	59	17%	7%	76%
StdMO	40 ng	44	0%	0%	100%
Xatv5misMO	40 ng	25	0%	0%	100%
XatvMO+tXAR[1]	40 ng+100 pg	46	28%	0%	72%
Anti-XleftyMO	2.5 ng	12	33%	67%	0%

Anterior defect: embryos that have small or no eyes. [1]We injected them into vegetal region of each blastomere at 4-cell stage.

was injected into embryos (Fig. 2F). The expression of *BF-1* seemed to be also affected in the region where linage tracer was not: the XatvMO might be diffusible and leak the other blastomere but not *β-gal* mRNA. However, *Krox20* expression was detected at normal levels in the hindbrain (Bradley *et al.,* 1993) of the XatvMO-injected embryos (Fig. 2F), suggesting that neural posterior structures were relatively unaffected by the depletion of *Xatv*. Immunostaining with Tor70, a monoclonal antibody specific for notochord (Bolce *et al.,* 1992), showed an enlarged notochord in the embryos injected with XatvMO (Fig. 2H). Finally, the expression of *XmyoD*, which is normally detected in the somite (Takahashi *et al.,* 1998), was expanded and ectopically induced in the embryos injected with XatvMO (Fig. 2J). These results indicate that the depletion of *Xatv* caused an expansion of caudal mesoderm, in turn inducing a posteriorization of the neural tissue.

We further analyzed the *Xatv*-depleted embryos to examine early mesodermal markers using whole-mount *in situ* hybridization

(Fig. 3). Once again, XatvMO or StdMO was coinjected with *β-gal* mRNA into one or two blastomeres of a 4-cell stage embryo. In zebrafish, loss-of-function studies of *wnt8* showed that it is required for neural posteriorization and that nodal signaling controls its expression (Erter *et al.,* 2001). In *Xenopus*, *Xwnt8* also acts as a caudalizing signal (Christian *et al.,* 1991). XatvMO-injected gastrulae had higher expression of *Xwnt8* (Fig. 3B) than StdMO-injected gastrulae (Fig. 3A). In early and mid gastrulae embryos injected with StdMO, dorsal and ventral regions of mesoderm formed in the marginal zone, as visualized by *Xbra* expression (Smith *et al.,* 1991). However, this pattern of restricted expression of *Xbra* was not seen in embryos injected with XatvMO, where expression was dispersed into the animal region (Fig. 3D). Expression of the dorsal mesodermal markers *Xbra*, *Xnot,* and *Chordin,* which delineate the dorsal midline including notochord (Sasai *et al.,* 1994, Smith *et al.,* 1991, von Dassow *et al.,* 1993), was elevated in the XatvMO-injected region, an observation that correlates with the enlargement of the notochord (Fig. 3 F,H,J). These results suggest that *Xatv* may control the precise formation of the trunk-tail organizer and may suppress any excess caudalization signals. *Xatv* transcription is induced by nodal signaling, suggesting that *Xatv* mRNA is a good marker to test for the activation of nodal signaling (Cheng *et al.,* 2000, Tanegashima *et al.,* 2000). StdMO did not affect the expression pattern of *Xatv* mRNA (Fig. 3K), whereas XatvMO increased it (Fig. 3L), suggesting that the enhanced expression of mesodermal genes is caused by up-regulation of nodal signaling.

Xatv inhibits EGF-CFC activity

EGF-CFC coreceptors have been implicated as being essential for nodal signaling. We next investigated whether *Xatv* could inhibit nodal signaling mediated by the *Xenopus* EGF-CFC gene *FRL-1* in animal cap assays (Fig. 4). Previous studies have shown that overexpression of *Xnr1* induces the expression of the mesodermal marker *Xbra*, the dorsal marker *chordin*, the endodermal marker *Mixer*, and *Xnr1* itself in animal caps (Engleka *et al.,* 2001, Jones *et al.,* 1995, Osada *et al.,* 2000, Zorn *et al.,* 1999). Whereas neither overexpression of *FRL-1* nor low doses of *Xnr1* could induce expression of these genes (Fig. 4A, lanes 2, 3), coinjection of *FRL-1* and a low dose of *Xnr1* were effective in inducing these markers (Fig. 4A, lane 4), suggesting that *FRL-1* enhanced the responsiveness to *Xnr1*. Conversely, *Xatv* inhibited the expression of these nodal response genes that were induced by coinjection of *Xnr1* and *FRL-1* (Fig. 4A, lane 5), suggesting that *Xatv* suppresses nodal signaling mediated by *FRL-1*. As previously reported, overexpression of *FRL-1* induced caudalized phenotypes when injected into the dorsal marginal zone of 4-cell stage embryos (n=33, 48%; Fig. 4B), whereas this phenotype was suppressed by the coinjection of *Xatv* (n=34, 85%; Fig. 4C). EGF-CFC genes have two distinct domains: an EGF-like motif and a CFC motif. A previous study showed that a cripto protein in which these two domains were mutated acted as a dominant-negative inhibitor (Yeo and Whitman 2001). We constructed two cDNA constructs encoding an EGF-like motif-deleted form with the flag-epitope tag (*FRL-1ΔE*) and a CFC motif-deleted form with the flag-epitope tag (*FRL-1ΔC*). *FRL-1ΔE* had no effect on nodal signaling enhanced by *FRL-1* (Fig. 4A, lane 6), but *FRL-1*ΔC was strongly inhibitory (Fig. 4A, lane 7), suggesting that it acts as a dominant-negative inhibitor of nodal signaling mediated by *FRL-1*. Embryos injected with *FRL-1ΔC* had very similar phenotypes to embryos injected with *Xatv*, such as a shortened axis (n=30, 100%; Fig. 4D;

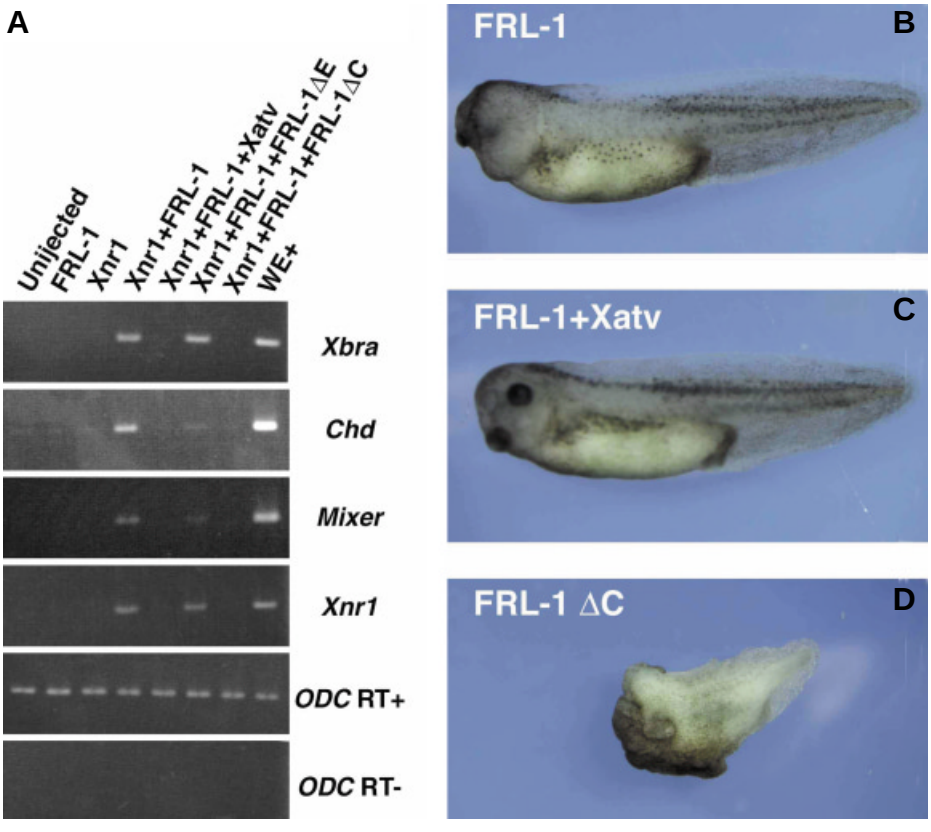

Fig. 4. *Xatv* inhibits the activity of *FRL-1*. (A) Xatv *inhibits nodal signaling enhanced by* FRL-1 *in animal caps. mRNA was animally injected into each blastomere of 4-cell stage embryos, whose animal caps were dissected at stage 9 and harvested at stage 10.5. The animal cap assay showed that 1 ng* FRL-1 *or 5 pg* Xnr1 *did not induce the expression of* Xbra, Chordin (chd), Mixer *and* Xnr1, *but the coinjection of* FRL-1 *and* Xnr1 *did induce the expression of these genes. One nanogram of* Xatv *inhibited the expression of these nodal-responsive genes induced by the coinjection of 5 pg* Xnr1 *and 1 ng* FRL-1. *This result suggests that* Xatv *inhibits the activity of EGF-CFC genes to suppress nodal signaling. In addition, the synergistic induction with* FRL-1 *was not inhibited by* FRL-1 *ΔEGF-like domain (* FRL-1 *ΔE) but was inhibited by* FRL-1 *ΔCFC domain (* FRL-1 *ΔC), suggesting it has a dominant-negative effect. (B-D)* FRL-1, FRL-1 *and* Xatv, *and* FRL-1*ΔC mRNA were injected into dorsal blastomeres of 4-cell stage embryos which were grown to tadpole stage.* **(B)** FRL-1 *(1 ng) induces caudalized phenotypes.* **(C)** Xatv *(2.5 pg) rescues the phenotypes caused by 1 ng* FRL-1. **(D)** FRL-1*ΔC (1 ng) induces the phenotype that is similar to overexpression of* Xatv.

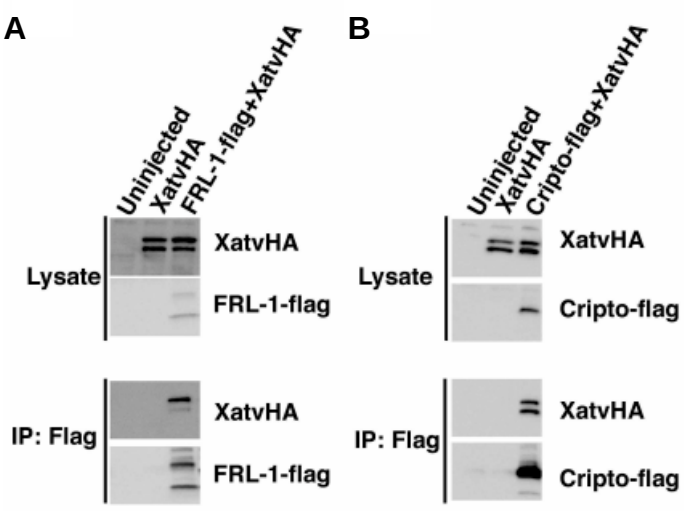

Fig. 5. Xatv interacts with EGF-CFC protein. mRNA *was injected into each blastomere of 4-cell stage embryos, which were cultured until stage 10.5. The proteins were extracted from injected and uninjected embryos with lysis buffer and loaded along with 5% of the total samples that were used for immunoprecipitation (IP).* **(A)** *FRL-1 protein interacted with Xatv. Embryos injected with either 1 ng* XatvHA*, or 1 ng each of* XatvHA *and* FRL-1-flag*, as well as uninjected control embryos were harvested at stage 10.5, and their lysates were immunoprecipitated with an anti-flag antibody. XatvHA protein was not immunoprecipitated from embryos injected with* XatvHA *alone, but was precipitated from embryos coinjected with* XatvHA *and* FRL-1 flag. **(B)** *Cripto protein interacted with Xatv. Embryos injected with either 1 ng* XatvHA *or 1 ng each of* XatvHA *and* Cripto-3flag*, as well as uninjected embryos, were used for the immunoprecipitation assays. XatvHA protein was immunoprecipitated from embryos that were coinjected with* XatvHA *and* Cripto-3flag.

(Tanegashima *et al.,* 2000). In conclusion, *Xatv* inhibits the activity of *FRL-1* to regulate nodal signaling.

Xatv binds to EGF-CFC proteins

Our results suggest that FRL-1 and Xatv antagonize each other in the regulation of nodal signaling. We used the co-immunoprecipitation assay to determine whether these two proteins interact. *XatvHA* mRNA was coinjected with or without flag-*tagged FRL-1* or *cripto* mRNA into *Xenopus* embryos, and then embryo lysate was used for immunoprecipitation with flag antibody. XatvHA was precipitated in the presence, but not the absence, of *FRL-1-flag* (Fig. 5A) or *cripto-3flag* protein (Fig. 5B). These results suggest that Xatv interacts with EGF-CFC gene products to suppress nodal signaling.

Discussion

Lefty, *antivin,* and related genes have been implicated as feedback inhibitors of *nodal* and its related genes in mesendoderm induction, anterior-posterior patterning, and left-right asymmetry during vertebrate embryogenesis (Schier and Shen 2000). Our results using an XatvMO suggest that proper formation of the trunk-tail organizer requires a balance between *Xnrs* and *Xatv*. The expression of mesodermal markers was up-regulated in XatvMO-injected gastrulae, suggesting an expansion of the trunk-tail organizer. This phenotype resembled that of embryos injected with *FRL-1* (Kinoshita *et al.,* 1995, Yokota *et al.,* 2003). We also found that *FRL-1* up-regulated the expression of genes that are controlled by nodal signaling, whereas coinjection of *Xatv* suppressed the activity of *FRL-1* in the animal cap. Furthermore, co-immunoprecipitation assays showed that *Xatv* could interact with EGF-CFC proteins FRL-1 and cripto. From these results, we propose that antagonism between *nodal* and *antivin* regulates anterior-posterior axis formation via the EGF-CFC proteins.

Negative regulation of nodal-related proteins in vertebrate development

In *Xenopus*, nodal signaling has been suggested to act as a posteriorizing signal by inducing caudal mesoderm (Piccolo *et al.,* 1999). *Cerberus-short form*, an inhibitor of nodal signaling, can induce head structures in the VMZ. Furthermore, pCS2+*Xnr1*-DNA was implicated in the induction of posteriorization and up-regulated the expression of the mesodermal gene *Xbra* (Piccolo *et al.,* 1999). In zebrafish, loss-of-function studies of *wnt8* showed that it is required for neural posteriorization and that nodal signaling controls its expression (Erter *et al.,* 2001). Here, we showed that an enlarged mesodermal structure was induced in embryos injected with XatvMO, leading to the up-regulation of *Xwnt8* expression. Our results, therefore, imply that *Xnrs* regulate induction of mesodermal tissue that in turn promotes caudalization, which is antagonized by *Xatv*.

Recent studies have shown that nodal antagonism is required for normal gastrulation in mouse, zebrafish, and *Xenopus* (Branford and Yost 2002, Feldman *et al.,* 2002, Perea-Gomez *et al.,* 2002). Previous studies of *nodal* mutant mice have suggested that the null mutation blocks gastrulation (Conlon *et al.,* 1994, Zhou *et al.,* 1993) but the hypomorphic mutant only shows defects of anterior-posterior patterning (Lowe *et al.,* 2001). In contrast to our results with XatvMO, another MO against a *lefty*/*antivin*-related gene in *Xenopus* (anti-XleftyMO) was reported recently to cause exogastrulation (Branford and Yost 2002); this difference is comparable to the difference seen between the null and hypomorphic mutants of *nodal* (Conlon *et al.,* 1994, Lowe *et al.,* 2001, Zhou *et al.,* 1993). We found that XatvMO inhibited *Xatv* translation when 100 pg *Xatv* mRNA was injected into embryos but not when 1 ng *Xatv* mRNA was injected (data not shown). This result suggested that XatvMO might have a relatively weak effect, as is seen in hypomorphic mutants. In fact, we also found that low doses of anti-XleftyMO caused posteriorization (Fig. 1H) without gastrulation defects. Here, we showed that antagonism between *nodal* and *lefty*/*antivin* is also required for proper anterior-posterior axis formation, which is a new example of *nodal* antagonism.

Xatv inhibits EGF-CFC activity to suppress nodal signaling

Lefty and *antivin* show a conserved function as antagonists of nodal signaling during various developmental processes (Schier and Shen 2000). However, the mechanism of inhibition of nodal signaling remains unknown. This report presents the first evidence of functional antagonism and biochemical interaction between a *lefty*/*antivin*-related protein and an EGF-CFC protein. Several

studies have indicated that the function of *lefty*, *antivin,* and their related genes is opposed to that of the EGF-CFC genes. In *cripto* mutant mice, the expression of some trunk organizer genes such as *brachyury* and *FGF8* was not detected at 7.5 days postcoitus (Ding *et al.,* 1998). In contrast, *lefty-2* mutant mice show increased expression of these genes (Meno *et al.,* 1999). Furthermore, the zebrafish maternal and zygotic *one-eyed pinhead* mutant (*MZoep*) is a phenocopy of *antivin*-overexpressing embryos (Meno *et al.,* 1999, Zhang *et al.,* 1998). These observations suggest that *lefty*, *antivin,* and their related genes may suppress the activity of EGF-CFC genes in vertebrate development. It is still unknown how Xatv acts to antagonize the activity of EGF-CFC protein to enhance nodal signaling. The simplest idea to account for Xatv function is that Xatv and nodal bind to cripto competitively. However, we found that there is no competitive binding between Xnr1 and Xatv (data not shown) and that Xatv did not bind to an EGF-like domain responsible for nodal binding (data not shown, Yan *et al.,* 2002). Two recent papers show the inhibitory action against activin or nodal signaling through the binding to cripto protein. First, tomoregulin inhibits nodal signaling through inhibition of interaction between Alk4 and cripto (Harms and Chang 2003). Second, the complex of cripto and activin plays an inhibitory role in activin signaling by complex formation with ActRII (Adkins *et al.,* 2003, Gray *et al.,* 2003). Our results suggest the possibility that a similar mechanism might be used for lefty/antivin action.

Materials and Methods

Embryos

Eggs were obtained by injecting human chorionic gonadotropin (Gestron: Denka Seiyaku, Japan) into *Xenopus*. Fertilized eggs were obtained by artificial insemination and dejellied using sodium mercaptoacetate. Microinjections were carried out according to Tanegashima *et al.,* (2000). In animal cap assays, mRNAs were injected into each blastomere of 4-cell stage embryos, whose animal caps were dissected at stage 9 using fine needles on 0.5 mm squares. These were cultured in 100% Steinberg solution containing 0.1% bovine serum albumin until sampling. Embryos were staged according to a previous study (Nieukoop and Faber 1956).

Constructs and mRNA preparation

For construction of pCS2-*Xatv*, the *Xatv* open reading frame (ORF) was amplified by polymerase chain reaction (PCR) with primers (forward, 5'-CACAGAATTCACATCAGAATGGGTGTCACTACC-3'; reverse, 5'-CACACTCGAGAACTTAGTGCTGCCATCTG-3'). PCR products were digested by *Eco* RI and *Xho* I and ligated into pCS2 vector. For the pCS2-*Xatv mutant (Xatvmut)* construct, silent nucleotide substitutions in the target nucleotide of XatvMO (ATGGGgGTaACcACt (*Xatv* +1 to +15): lower case: substitutions to XatvMO) were introduced into the pCS2-*Xatv* construct by PCR without changing any other region of the construct. For the *XatvHA* and *XatvHAmut* constructs, pCS2-*Xatv* and *Xatvmut* were digested by *Nde* I, blunt-ended, and then digested by *Eco*RI to release an ORF without a stop codon. Released fragments were ligated with HA tag (5'-TATCCGTATGATGTTCCTGATTATGCTTGACtcga-3': lower case letters indicate a 5' sticky end generated by *Xho*I digestion) into pCS2 vector. To construct pCS2-*FRL-1-flag*, we digested pCS2-*FRL-1-6myc* (Yabe *et al.,* 2003) with *Cla* I to release FRL-1ORF without a stop codon, and blunt-ended the fragment, which was ligated into the pCS2-*3flag* vector. pCS2-*FRL-1*ΔCFC-flag and pCS2-*FRL-1*ΔEGF-flag constructs encoded the FRL-1-flag protein without a CFC domain (113Pro-150Asp) or EGF domain (77Lys-112Arg), respectively. All constructs were sequence verified. For the preparation of mRNA, pCS2-nuclear localizing signal-*β-gal*/ (Takahashi *et al.,* 2000), pCS2-*Xatv*, pCS2-*Xatvmut*, pCS2-*XatvHA*, pCS2-*XatvmutHA*, pCS2-*Xnr1* (Sampath *et al.,* 1997), pCS2-*FRL-1* (Yabe *et al.,* 2003), pCS2-*FRL-1-flag*, pCS2-*FRL-1*ΔCFC-flag, pCS2-*FRL-1*ΔEGF and pCS2-*Cripto-3flag* (Yeo and Whitman 2001) were all linearized using *Not*I. These templates were transcribed using the mMESSAGE mMACHINE SP6 kit (Ambion). *Not*I-linearized pCS2-*Xnr1* (Sampath *et al.,* 1997) was used for DNA injection.

Morpholinoantisense oligonucleotide

Morpholinoantisense oligonucleotide against *Xatv* (XatvMO) and StandardMO (StdMO) were designed as described below: XatvMO: 5'-AAAGATTTGGTAGTGACACCCATTC-3'; bold letters indicate the first codon. Xatv5misMO: 5'-AAAcATTTcGTAaTGACACgCAaTC-3'; small letters indicate changes to the XatvMO sequence. StdMO: 5'-CCTCTTACCTCAGTTACAATTTATA-3' (Gene Tools, LLC). The oligonucleotide sequence used for anti-XleftyMO was as previously reported (Branford and Yost 2002).

Histology

Histologic analyses were performed exactly as described in Tanegashima *et al.,* (2000).

RT-PCR

Total RNA isolation, reverse transcription-polymerase chain reaction (RT-PCR) method and the primer pairs used here for *Xbra*, *Chordin,* and *ornithine decarboxylase* (*ODC*) were as described in Tanegashima *et al.,* (2000). Primer sets for *Xnr1* and *Mixer* were as previously described(Hayata *et al.,* 2002, Jones *et al.,* 1995). *ODC* was used as a loading control. Reverse transcriptase-negative (RT-) reactions were included to show the absence of genomic DNA contamination.

Whole-mount in situ *hybridization*

Whole-mount *in situ* hybridization was carried out using digoxigenin-labeled antisense probes (Harland 1991). For cell-lineage tracing, we injected albino embryos with 250 pg *β-gal* mRNA. Injected regions were stained with red-gal (Research Organics, Inc) in the reaction buffer (1 mM $MgSO_4$, 10 mM $K_3Fe(CN)_6$, 10 mM $K_4Fe(CN)_6$, 0.1 M phosphate buffer, and 0.1% Triton X-100). Probes were synthesized using pBluescript-*Chordin* (Sasai *et al.,* 1994), pBluescript-*Xatv* (Tanegashima *et al.,* 2000), pBluescript-*Xnot* (Tanegashima *et al.,* 2000), pXT1-*Xbra* (Smith *et al.,* 1991), pCS2-*BF-1* (Bourguignon *et al.,* 1998), pBluescript-*XmyoD* (Takahashi *et al.,* 1998), and pGEMT-*Krox20* as templates. pGEMT-*Krox20* was originally obtained by cloning PCR products.

Whole-mount in situ *immunohistology*

Albino embryos were fixed in MEMFA (0.1 M MOPS (pH 7.4), 2 mM EGTA, 1 mM $MgSO_4$, 3.7% formaldehyde) for 1 hour at room temperature. Tor70-antiserum (kindly provided by R. Harland) was used at a dilution of 1:5. The secondary antibody, alkaline phosphatase-conjugated goat anti-mouse IgG + IgM (Biosource International) was used at a dilution of 1:5000. Signal was detected using BM purple (Roche). After detection, the stained embryos were cleared using Murray's solution (a 1:2 mixture of benzyl alcohol and benzyl benzoate).

Immunoprecipitation and Western blotting

In Fig. 1, the manually defollicled oocytes injected with *XatvHA* mRNA were cultured for 2 days in OR2 medium (82.5 mM NaCl, 2.5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM Na_2HPO_4, 5 mM HEPES-NaOH [pH 7.8]). Frozen oocytes were lysed in solubilization buffer (65 mM Tris-HCl [pH 7.5], 10 mM EGTA, 1 mM EDTA, 1 mM Pefablock SC [Roche], 1 μg/mL leupeptin [Roche]), and lysates were centrifuged to remove yolk protein. Samples were reduced using 100 mM dithiothreitol (DTT) and loaded onto sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels (1.5 oocytes/). For immunoprecipitation, the embryos were lysed by lysis buffer (137 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 8.1 mM $Na_2H PO_4$ [Dulbecco

phosphate-buffered saline] supplemented with 1% Triton, 1 mM Pefablock SC [Roche] and 1 μg/mL leupeptin [Roche]), and centrifuged to remove yolk protein. For the immunoprecipitation assay, the lysates were treated with protein G-Sepharose (Amersham Bioscience) bound with anti-flag monoclonal antibody M2 (Sigma). Tagged proteins were detected using anti-HA-peroxidase high affinity antibody (Roche) and a rabbit polyclonal anti-flag antibody (Sigma). For loading controls, membranes were reprobed with mouse monoclonal α-actin antibody (Sigma).

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