Cooperative non-cell and cell autonomous regulation of Nodal gene expression and signaling by Lefty/Antivin and Brachyury in *Xenopus*

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Received for publication 2 August 2005, revised 20 October 2005, accepted 28 October 2005

Available online 6 January 2006

Abstract

Dynamic spatiotemporal expression of the *nodal* gene and its orthologs is involved in the dose-dependent induction and patterning of mesendoderm during early vertebrate embryogenesis. We report loss-of-function studies that define a high degree of synergistic negative regulation on the *Xenopus nodal*-related genes (*Xnrs*) by extracellular *Xenopus antivin/lefty* (*Xatv/Xlefty*)-mediated functional antagonism and Brachyury-mediated transcriptional suppression. A strong knockdown of *Xlefty/Xatv* function was achieved by mixing translation- and splicing-blocking morpholino oligonucleotides that target both the A and B alloalleles of *Xatv*. Secreted and cell-autonomous inhibitors of *Xnr* signaling were used to provide evidence that *Xnr*-mediated induction was inherently long-range in this situation in the large amphibian embryo, essentially being capable of spreading over the entire animal hemisphere. There was a greater expansion of the Organizer and mesendoderm tissues associated with dorsal specification than noted in previous *Xatv* knockdown experiments in *Xenopus*, with consequent exogastrulation and long-term maintenance of expanded axial tissues. *Xatv* deficiency caused a modest animal-ward expansion of the marginal zone expression territory of the *Xnr1* and *Xnr2* genes. In contrast, introducing inhibitory *Xbra-EnR* fusion constructs into *Xatv*-deficient embryos caused a much larger increase in the level and spatial extent of *Xnr* expression. However, in both cases (*Xatv/Xlefty*-deficiency alone, or combined with *Xbra* interference), *Xnr2* expression was constrained to the superficial cell layer, suggesting a fundamental tissue-specific competence in the ability to express *Xnrs*, an observation with direct implications regarding the induction of endodermal vs. mesodermal fates. Our experiments reveal a two-level suppressive mechanism for restricting the level, range, and duration of *Xnr* signaling via extracellular inhibition by *Xatv/Xlefty* coupled with potent indirect transcriptional repression by *Xbra*.

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Keywords: Morpholino; *Xnr*; *Xlefty/Xantivin*; *Xbrachyury*; Organizer; Mesendoderm induction; Gastrulation; *Xenopus laevis*

Introduction

Intercellular signaling via the TGFβ family member Nodal plays a central role in mesendodermal fate specification and patterning of vertebrate embryos (Schier, 2003; Schier and Shen, 1999; Whitman, 2001). Fundamental questions related to how the activity of this potent inducer is deployed during embryonic patterning include how it is regulated transcriptionally and post-transcriptionally, and how post-translational modifications affect protein secretion, ligand maturation from the pro-protein, or ligand movement characteristics within the extracellular milieu (Constam and Robertson, 1999; Le Good et al., 2005). Biological effects on the receiving cell also depend upon the availability of obligate receptor complex cofactors that are involved in signal receipt and transduction, such as the EGF-CFC proteins (Shen and Schier, 2000; Schier, 2003).

Little is known about whether the signals that initiate *nodal* expression are conserved across species, although links to maternally deposited inducers have been made in *Xenopus* and zebrafish (reviewed in Whitman, 2001; Schier, 2003). In several species, however, conserved *cis*-regulatory regions are beginning to be characterized that are involved in controlling later aspects of the maintenance and upregulation
of expression of nodal/nodal-related genes. Among these, an important mechanism for maintaining and upregulating nodal expression during gastrulation stages, when anteroposterior and mesendodermal cell fates are being specified, involves a FAST/FoxH1-dependent Nodal autoregulatory loop (Norris and Robertson, 1999; Adachi et al., 1999; Osada et al., 2000; Pogoda et al., 2000; Saijoh et al., 2000). Such loops have an intrinsic property of tending to expand expression of the autoregulated gene through embryonic tissue, an effect that must be offset by negative feedback mechanisms in order to prevent inappropriate inductive effects. Well known examples of such feedback in other signaling systems include the induction of Ptc by Hh (Ingham and McMahon, 2001), and Dad by the Drosophila TGFβ-related molecule Dpp (Tsuneyzumi et al., 1997).

An important extracellular feedback inhibitor of Nodal signaling is Lefty (also previously known as Antivin in frogs and zebrafish), whose transcription is directly activated by Nodal signaling (Meno et al., 1996, 1997, 1999; Bisgrove et al., 1999; Thisse and Thisse, 1999; Cheng et al., 2000; Tanegashima et al., 2000). Like Nodal, Lefty ligands are also released by protease cleavage from precursor pro-proteins (Sakuma et al., 2002). At the level of analysis carried out so far, Lefty is thought to work as a monomer to inhibit Nodal signaling at the level of the receptor complex, either by binding the EGF-CFC factor directly (Cheng et al., 2004; Tanegashima et al., 2004), or perhaps by physically interacting with the Nodal ligand itself (Chen and Shen, 2004). The idea that Lefty/Antivin is a key negative feedback regulator of nodal/Xnr expression fits well with their spatiotemporal expression characteristics. Loss-of-function experiments involving genetic manipulations in mice (Meno et al., 1999, 2001) or translational inhibition in frogs and zebrafish (Agathon et al., 2001; Chen and Schier, 2002; Feldman et al., 2002; Branford and Yost, 2002; Tanegashima et al., 2004) lead to increased and expanded Nodal signaling.

There is evidence from overexpression experiments in Xenopus and zebrafish that Lefty can move a long distance to suppress expression of Nodal-responsive genes in the marginal mesendodermal territory (Chen and Schier, 2002; Branford and Yost, 2002). Consistent with this idea, studies with mouse Nodal-GFP and Lefty-GFP fusion proteins in chicken embryos suggest that both ligands move relatively far, but that Lefty travels farther and faster than Nodal (Sakuma et al., 2002). The latter relationship is a tenet of reaction-diffusion models for inducer/antagonist signaling loops in embryonic patterning (Turing, 1952; Gierer and Meinhardt, 1972; Meinhardt and Gierer, 2000; Juan and Hamada, 2001; Chen and Schier, 2002). Because the relative level of Nodal signaling output arising from Nodal-Lefty (Xnr-Xatv/Xlefty) antagonism is likely to be a key determinant of cell fate, important goals include a full mechanistic description of the factors that affect the expression, diffusibility, and perdurance of Nodal and Lefty/Antivin in vivo.

The previous Xlefty/Xantivin (Xatv) loss-of-function studies in Xenopus (Branford and Yost, 2002; Tanegashima et al., 2004) showed less extensive effects on marker gene expression than those observed in zebrafish or mouse embryos. However, there was significant expansion in the expression of several mesendodermal markers, and of Xnr2, although the latter still remained relatively well restricted to the marginal zone region. The single antisense morpholino oligonucleotides (MOs) used in those papers targeted either the A or B copies (alloalleles) of Xlefty/Xatv that are found in this allotetraploid species. In our own studies, initiated before those papers were published, we reached similar conclusions regarding the effects of single MOs, but found variable phenotypes between batches of embryos, and thus became concerned with determining the effect of further reducing Xatv function by concurrently inhibiting both alloalleles. We report here that this manipulation produced a much more dramatic effect on downstream target gene activation and embryonic patterning, which was associated with an increased level and territory of Xnr expression and signaling. In agreement with previous studies, mesendodermal specification and patterning during early gastrulation in XatvMO-injected embryos began normally, but became greatly disrupted shortly thereafter. The abnormal embryonic morphogenesis was associated with very broad expansion of mesendodermal marker gene expression, including for example expansion of Xbra from the normal equatorial band to cover almost the entire animal cap region. A failure of involution combined with abnormal convergence/extension movements led to a form of exogastrulation, involving hyperdorsalization (overspecification of mesendodermal tissue), a morphant phenotype that is reminiscent of Xnr-induced hyperdorsalization. Our analysis provides stronger evidence that Xatv is a potent restrictor of the strength, duration, and range of Xnr signaling during the cell fate specification and patterning that occurs prior to and during gastrulation. Results with Xnr-selective inhibitors indicate that the massive expansion of the organizer and mesendodermal territory observed after Xatv knockdown was caused by large-scale increases in Xnr signaling range, which for certain genes extended to cover the entire animal hemisphere of this large embryo. These alterations of Xnr signaling during gastrulation caused enlargements of the mesendodermal tissues that were maintained in later stage embryos. Moreover, we found that the relatively small spatial expansion of Xnr expression in XatvMO-injected embryos is linked to an indirect transcriptional suppression from the expanded Xbra domain. The latter finding strongly supports the previous notion (Kumano et al., 2001) of a fundamental level of cross-regulation between Xbra and Xnr, but leads to a new understanding of the dual level regulation that ensures the transient and restricted nature of Xnr expression during gastrula stages. The expanded Xnr2 expression domain in XatvMO- and/or Xbra-EnR-injected embryos is remarkably restricted to the superficial layer of the embryo, suggesting that the embryonic tissues exhibit a differential competence with respect to initiating the expression of essential regulatory genes such as the Xnrs. We present an integrated model for the multiple influences that cause the expression of Xnrs to be initiated and maintained in a narrow domain of the superficial marginal zone, which is a key determinant of their overall
inductive range for mesendodermal fate specification during gastrulation stages.

Materials and methods

Embryo manipulation and microinjections

Embryos were obtained by in vitro fertilization, de-jellied with 1% thioglycolic acid solution and cultured in 1× Steinberg’s solution (SS; Kay and Peng, 1991) at 22°C, and staged according to Nieuwkoop and Faber (1967). Embryos were microinjected in 1× SS containing 5% Ficoll and then transferred and maintained in 1× SS. Capped mRNAs for microinjection were prepared using mMESSAGE mMACHINE (Ambion) according to the manufacturer’s instruction with templates from the following linearized plasmids: pCS2+Xnr2 (Jones et al., 1995); pCS2+CerS (Piccolo et al., 1999); pSP64T-tALK4 (Chang et al., 1997); pSP64T-Xbra-EnK (Conlon et al., 1996); pCS2+nl/gal; pBSKII-Xatv*. For inducing exogastrulation from normal embryos, vitelline membranes were removed from embryos at stage 8 and embryos were incubated in High Salt 1× SS (100 mM NaCl final concentration; all other components standard) until sibling embryos completed gastrulation. After high salt treatment, embryos were changed to 0.1× SS and collected at stage 25. XatvMO1-injected embryos were incubated in 0.1× SS without vitelline membranes from stage 8 to the end of gastrulation and transferred to 0.1× SS until stage 25.

Morpholino oligonucleotides (MOs)

XatvMO1 (5′-ACCCATTCTGTGACGTCTACA-3′) was designed complementary to the region around the Xatv translational start site (Fig. 1A). XatvMO2 (5′-AGGACTTGAAATACCTGCATTGCCC-3′) was generated from Xatv exon1/intron1 sequences determined from a genomic lambda phage clone using PCR (Fig. 1A); it is currently uncertain if Xatv MO2 is a transcript specific to Xatv exon1/intron1 sequences. The control morpholino was Gene Tools V-CCTCTTACCTCAGTTACAATTTATA-3 (Conlon et al., 1996); pCS2+miRNA/5′-UTR and coding region of XatvMO1-target sequences in the 5′-UTR of pCS2 + XatvMO2 was constructed by adding 5 nucleotides into 5′-UTR of pCS2 + XatvMO2 using quick change PCR site-directed mutagenesis, resulting in the exactly same sequence as 5′-UTR of Xatv. Therefore, cDNAs of two Xats in plasmid constructs contain the same coding sequences (Fig. 1A). In vitro translation was performed as described (Taylor et al., 1996) with some modifications. Each RNA (1 μg) was mixed with 1 μg of the specified MO, heated to 70°C, slowly cooled to 37°C, then added to nuclease-treated rabbit reticulocyte lysate (Promega) with Redivue Pro-mix [35S] labeling mix (>1000 Ci/mmol; Amersham) and incubated for 1 h at 30°C. Xnr2 translation was used as a negative control.

Construction of Xatv cDNA

The specificity of XatvMO1 was tested by restoration of embryonic patterning by Xatv rescue cRNA (Xatv*). Quick-change PCR site-directed mutagenesis (Stratagene) was used on pBSKII-Xantivin (Xatv; Cheng et al., 2000) to delete/alter XatvMO1 target sequences in the 5′-UTR and coding region with oligonucleotides: 5′-GGCACTTGACCCGAGGTGGCTACTACTA-CAATTAC-3′; 5′-AGATTGGATGACCGCCATACGGGTGCAAGTCCC-3′ (ATG underlined; see Fig. 1A for final sequence of rescue RNA). Modifications were confirmed by sequencing. The specificity of the splicing blocker XatvMO2 was shown by rescue with cojected “wild-type” Xatv cRNA.

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Fig. 1. Specific inhibition of Xatv translation and splicing with morpholino oligonucleotides. (A) Xatv MO positions relative to the translational start sites on Xatv mRNAs (XatvMO1 and leftyB-MO) and the exon 1/intron 1 junction on Xatv genomic DNA (XatvMO2); see Materials and methods for actual MO sequences (reverse complements of those shown here). (B) XatvMO1 inhibits the translation of Xatvδ in vitro. While translation of Xatvδ is completely inhibited by XatvMO1, Xatvδ translation is not blocked (lane 4). Neither control MO nor XatvMO2 affects translation of Xatv mRNAs (lanes 3, 5). Translation of Xnr2 RNA is used as both loading control and negative control. (C) XatvMO2 specifically inhibits Xatv pre-mRNA splicing (embryos received 50 ng of each XatvMO and were collected at the indicated stages). Primers in panel A (arrows) amplify across intron 1 using unspliced Xatvδ and Xatvδ pre-mRNA. (D) XatvMO1 preferentially inhibits Xatvδ pre-mRNA splicing. Embryos injected with 50 ng of XatvMO2 were collected at stage 10.5. RT-PCR was performed with A or B primer sets that amplify Xatvδ or Xatvδ pre-mRNA, respectively; see Materials and methods for primer sequences.
Whole-mount in situ hybridization and lineage tracing

Whole-mount in situ hybridization was performed as described (Sive et al., 2000). Embryos were fixed with 1× MEMFA (1 h). Some fixed embryos were embedded in 2% low melting agarose and bisected before or after in situ hybridization with a razor blade. Antisense probes were synthesized using Dig-labeling kit (Roche) from linearized plasmids: pBSK-Xnr2 (Jones et al., 1995); pBSKII-Gae (Cho et al., 1991); pCS2+Chordin (Sasai et al., 1994); pCR-Script-ADMP (Moos et al., 1995); pCS2+Cer (Piccolo et al., 1999); pBSKII-XAntivin (Cheng et al., 2000); pXT1-Xbra (Smith et al., 1991); pBS-Derrière (Sun et al., 1999); pCS2-XWnt8 (Sokol et al., 1991); pT7Blue-3-Mixer (Henry and Melton, 1998); pSTBlue-1-Sox17a (Hudson et al., 1997); pBSK-mp-1 (Richter et al., 1988); pBSKs-ctl-1 (Richter et al., 1988); pBSKS-ots2 (Blitz and Cho, 1995); pBS-Xkrox20 (Bradly et al., 1993); pfhh4-Shh (Ruiz i Altaba et al., 1995); pBS-Edd (Sasai et al., 1996); pBSKS-MyoD (Hopwood et al., 1989). Hybridization was revealed using alkaline phosphatase conjugated color substrate. For lineage tracing, embryos were coinjected with anti-digoxigenin Fab fragments (Roche) and BM purple (Roche) as nuclear stain. Histological analysis was performed as described in Cheng et al. (2000), except using Histo-clear (National Diagnostics) instead of toluene. After transverse section of three high salt (HS) or XatvMO2-induced exogastrulae, seven serial sections per each exogastrulae were selected from the middle region of the mesendodermal mass with respect to the A/P axis. Areas of notochord or somite from whole exogastrulae were determined by weighing prints of the digital images and expressed as mean percentage ± SE. Significace of the tissue area alterations between HS- and XatvMO2-induced exogastrulae used the unpaired t test.

Results

Morpholino-based inhibition of translation and splicing of Xenopus Lefty/Antivin

We adopted a morpholino oligonucleotide-based approach to block the function of both XatvA and XatvB alloalleles, concurrently, to gain a full appreciation of the role of Xatv in limiting Xnr signaling range during Xenopus embryogenesis. A translation-inhibiting MO, XatvMO1, was designed from 5'-UTR and translational initiation sequences present in 24 gastrula stage Xatv cDNAs (Cheng et al., 2000) of the A and B alloalleles (at approximately 2:1 ratio). The two alleles have previously been referred to as Xlefty A and Xlefty B (Branford et al., 2000; Fig. 1A). The 25 nt XatvMO1 matches XatvA over its entire length and XatvB over the first 18 nt of the MO 3' end (Fig. 1A). The current understanding of MO function (GeneTools Inc., technical advice) suggests that XatvMO1 should suppress translation of XatvA and XatvB mRNAs. However, XatvMO1 failed to block in vitro translation of XatvB cRNA under conditions that effectively blocked XatvA translation (Fig. 1B, lane 4). Translation of the rescue RNA Xatv* (Fig. 1A) used in the specificity tests below was not affected by XatvMO1 (Fig. 1B, lane 4). Translation of Xnr2, a negative control, was not affected by any XatvMO that we tested (Fig. 1B), and the GeneTools control MO did not affect translation of any RNA. Concerns that the unbound 3' tail of XatvMO1 might facilitate its displacement from XatvB mRNA led us to synthesize another MO, XatvMO2, to target the splicing of XatvB pre-mRNA at the exon1/intron1 junction (Fig. 1A).

The splice blocker XatvMO2 did not reduce translation of XatvA* or XatvB* RNA (Fig. 1B, lane 5). The efficacy of splicing blocking by XatvMO2 was assessed by RT-PCR using primers to amplify across intron 1 (Fig. 1A) of both XatvA and XatvB on template RNA extracted from XatvMO2-injected (50 ng, 1-cell stage) gastrula stage embryos. This method detects spliced and unspliced RNA without regard for its derivation from XatvA and XatvB. While spliced RNA was still detected, substantial unspliced pre-mRNA was detected in XatvMO2-injected embryos compared to controls (Fig. 1C). The aggregate amount of [unspliced + spliced] RNAs was increased compared to controls, likely related to enhanced Xatv expression induced by the increased Xnr signaling in the Xlefty/Xatv-deficient situation (see below), with perhaps some contribution from MO-induced mRNA stabilization. Next, to analyze the selectivity of XatvMO2 against splicing of RNA from the XatvA or XatvB alloallele, RT-PCR was performed with primers designed based on cDNA sequences that are selective for XatvA or XatvB RNA. XatvA and XatvB RNAs were expressed at similar levels in normal embryos, consistent with previous reports (Fig. 1D, lanes 3 and 4; Branford et al., 2000). In XatvMO2-injected embryos, however, spliced PCR products are mostly XatvA-class (Fig. 1D, lane 5). XatvB mRNA splicing was blocked to a small degree, as indicated by the presence of the slightly larger “unspliced” DNA fragment compared to XatvB (Fig. 1D, lane5); whether this is because there is increased Xatv/Xlefty expression because of the increased Xnr signaling is currently unknown. There was, in contrast, a major effect on XatvB splicing (Fig. 1D, lane 6). The selective effect of XatvMO2 on XatvB splicing was confirmed by testing the PCR products generated in the same way as Fig. 1C for cleavage by XatvB-specific restriction enzymes, chosen from XatvB exon and intron sequence (the XatvB intron sequence is not available yet). In pilot experiments, they did not cleave...
intron-spanning DNA fragments made by RT-PCR with XatvA-specific intron-flanking primers (data not shown). Overall, these data demonstrate that XatvMO1 mainly inhibits translation of XatvA mRNA, and that XatvMO2 selectively and efficiently targets the splicing of XatvB pre-mRNA.

**Overall embryonic defects and specificity of XatvMO function**

We first compared the Xatv loss-of-function phenotype after injecting XatvMO1, XatvMO2, or both mixed together at the 1-cell stage (Fig. 2A). Experimental embryos scored at late
neurula to tailbud stages displayed a spectrum of defects from severe gross exogastrulation, through a typical phenotype associated with abnormal gastrulation – an “open-back” defect in which the neural plate is split open – and down to almost normal tailbud morphology. Individual MOs caused a relatively low incidence of exogastrulation at the 50 ng dose (XatvMO1: 12%, n = 32; XatvMO2: 23%, n = 44). We tested whether concurrently blocking the function of XatvA and XatvB shifted the proportional representation of defects towards the more severe phenotype. The 35 ng dose of each individual MO produced a high incidence of open-back embryos. A mixture of a lower amount (25 ng each) of XatvMO1 + XatvMO2 induced a high proportion of exogastrulae (Fig. 2A). For unknown reasons, the XatvMO2 splice blocker alone induced the open-back phenotype at a lower incidence than the XatvA translation blocker XatvMO1 alone. A similar increase in effectiveness of XatvMO1 + XatvMO2 coinjection was seen by analyzing the expansion of mesendodermal marker expression (Figs. 3A: c, d, 4A: g–l; Supplemental Fig. 1A) in which Xnr2, Gsc, Xatv, and Xbra expressions were more expanded than in single MO injection. These data indicate a strong synergistic effect caused by simultaneously targeting XatvA and XatvB RNAs. During our studies, another report on MO-based loss-of-function of Xlefty was published by Branford and Yost (2002), who used XleftyA- or XleftyB-specific MOs in single injections. We synthesized XleftyB-MO and compared it alone, or in various mixtures with our MOs, to assess the knockdown effects. The 25 nt XleftyB-MO exactly matches the 5′-UTR of XatvB mRNA (Fig. 1A), and XatvA over 17 nt at the MO’s 3′ end with a gap of 6 nt mismatch and 2 end matches. Like the single injection of XatvMO1 or XatvMO2, no exogastrulae were caused by 35 ng of XleftyB-MO, although it caused ~100% incidence of the open-back phenotype (Fig. 2A). In contrast, combinations of MOs produced exogastrulae. Furthermore, the exogastrula incidence was higher in MO combinations that inhibit XatvMO1/XatvMO2 together than for XatvMO1/XleftyB-MO mixtures that mainly target only XatvMO1 (Fig. 2A). The incidence of the exogastrula by MO combinations that inhibit XatvMO1/XleftyA and XatvMO2/XleftyB together is comparable to that obtained by injecting approximately 70 ng of one Xlefty-MOs (40%; see Branford and Yost, 2002). The general view from marker analysis, however, suggested that combination MO-based loss of function of XatvMO1 and XatvMO2 caused much more profound effects on embryonic patterning than those targeting either alloallele. First, the expanded expression of specific markers, such as Xatv, was greater with XatvMO1 and XatvMO2 mixed MOs (e.g., Supplemental Figs. 1B: d, e), and the expansion was more reproducible across batches of embryos, with at least a 17–30% higher incidence of expanded Xatv expression during gastrulation. (A) XatvMO1 + MO2-injection upregulates and maintains Xnr1 and Xnr2 expression during gastrulation. (a–l) In situ hybridization (vegetal pole views) with Xnr2 in (a, c, i) uninjected or (b, f, j) control MO (60 ng)-injected embryos compared to those injected with (c, g, k) XatvMO1 alone (60 ng). Quantitative analysis of alterations shown: c, n = 5/5; g, n = 6/6; k, n = 8/8. (d, h, l) XatvMO1 + MO2 (30 ng each; quantitation: d, n = 14/15; h, n = 10/13; l, n = 13/13). Note the stronger Xnr2 signal in XatvMO1 + MO2-injected embryos than in XatvMO1-injected embryos. (m) RT-PCR with total RNA from whole embryos injected with 60 ng control MO, or 30 ng of each XatvMO1 and XatvMO2 (– RT, + RT controls are from uninjected embryos). (B) Xatv expression is increased and expanded in XatvMO1 + MO2-injected embryos during gastrulation. (a–c) Vegetal views. (d–I) Dorsal views. [Quantitation: c, n = 13/15; i, n = 21/23; l, n = 13/15]. (m, n) XatvMO1 + MO2-injected embryos at St. 12.5 were bisected longitudinally through the center of the Xatv expression domain after whole-mount in situ hybridization and viewed either (m) internally or (n) externally (green arrowheads, indentation of the superficial layer (see text); red arrowheads, epiboly margin).
expression alterations caused by the XatvMO1/XatvMO2 mixture led to its use for the experiments described below.

Next, we analyzed morphological changes in XatvMO1 + MO2-injected embryos. Compared to uninjected embryos, MO-injected embryos (either XatvMO1 + MO2 or control MO) were approximately 30–40 min delayed in initiating formation of the dorsal lip (data not shown), but from stage 10.5 onwards, control MO and uninjected embryos had indistinguishable rates of development. The great majority of XatvMO1 + MO2-injected embryos developed very similarly to control embryos until stage 10.5, forming an incipient dorsal lip and initial blastopore groove. As reported previously (Branford and Yost, 2002), lateral spreading of the blastopore lip stopped fairly abruptly at stage 10.5, and gastrulation movements were effectively aborted. At stage 18, the severely disrupted embryos lacked a proper dorsoventral or anteroposterior axis (Fig. 2B: c), somewhat similar to that caused by Xnr2 overexpression (Fig. 2B: d). Culturing of XatvMO1 + MO2-coinjected embryos when unconstrained by the vitelline membrane allowed a form of exogastrulation (Fig. 2C: b, d; also see Branford and Yost, 2002).

The XatvMO phenotype was specifically attributable to decreased Xatv function. When XatvMO1 was injected into 1-
cell embryos along with increasing doses of *Xatv* RNA (the 18 nt matching with *Xatv* MO1 was deleted, plus mismatches introduced; Fig. 1A), the phenotype was progressively rescued, leading to high proportions of normal embryos when scored at late neurula stage (Fig. 2D: a) or later (not shown). We note that the pBluescript-derived *Xatv* RNA is likely relatively inefficiently translated than when produced from other vectors, and *Xatv* did not cause a global embryonic phenotype in injected normal embryos. Because *Xatv* MO2 inhibits *Xatv* splicing but not translation (Fig. 1B, C), normal *Xatv* cRNA should overcome the defects caused by *Xatv* MO2. Wild type *Xatv* RNA dose-dependently rescued the defects caused by *Xatv* MO1 (data not shown) and the splicing blocker *Xatv* MO2 (Fig. 2D: b).

**Xatv depletion increases and prolongs Xnr expression**

To characterize further the role of *Xatv* in regulating *Xnr* signaling, we examined the expression patterns of various markers, including *Xnrs* themselves, in *Xatv* MO1 + MO2-injected embryos during gastrulation. We confirmed the previous results (Branford and Yost, 2002) with single MO injections (~50 ng), of a relatively modest effect on pan-mesodermal or region-specific (e.g., organizer) markers. In contrast, coinjection of *Xatv* MO1 + MO2 (25–30 ng each) into 1-cell embryos produced more dramatic alterations (Figs. 3A, 4A; Supplemental Fig. 1A). Because *Xatv* is thought to be a feedback inhibitor of *Xnr* autoregulation, we first examined the expression of the *Xnr* genes that are expressed in a localized manner during gastrulation and have been determined to be largely affected by intercellular *Xnr* signaling (Jones et al., 1995; Joseph and Melton, 1997; Takahashi et al., 2000). By RT-PCR (Figs. 3A: m) and whole-mount in situ analysis (*Xnr2*, Fig. 3A; *Xnr1*, Supplemental Fig. 2), expression of both genes became upregulated starting at around mid-gastrula stage, with noticeably increased transcript levels maintained during gastrulation. Together with the elevated expression level, *Xatv* depletion induced, as previously reported (Branford and Yost, 2002), a modest expansion towards the animal pole of the domain of *Xnr1* and *Xnr2* expression (Fig. 3A: a–d, 8C, C’; see Supplemental Fig. 2 for *Xnr1*; note that *Xnr1* transscripts are more difficult to detect and record than other *Xnr* genes). A significant difference in the level of *Xnr* (Fig. 3A: m) and *Xnr*-responsive gene expression from the mid-gastrula stage (Supplemental Fig. 1A) suggests that *Xatv* negative feedback regulation, even during relatively early gastrulation, is critical for achieving an appropriate level of *Xnr* signaling. Among the four remaining *Xnr* genes, *Xnr5* and *Xnr6* are primarily activated by maternal factors and not by intercellular *Xnr* signaling (Takahashi et al., 2000; Yang et al., 2002; Rex et al., 2002). Indeed, RT-PCR assays showed that the expression levels of both of these genes were unaffected during both blastula and gastrula stage (Fig. 3A: m). The expression level of *Xnr4*, which is maintained by intercellular *Xnr* signaling (Joseph and Melton, 1997; Agius et al., 2000), was unaffected in the *Xatv* MO1 + MO2-injected embryos until stage 10.5 (Fig. 3A: m). At stage 11.5, however, *Xnr4* showed a large increase compared to controls (Fig. 3A: m), likely related to its expression in axial midline tissue, which as described below is increased in *Xatv* MO1 + MO2-injected embryos (Fig. 6). *Xnr3* expression was not analyzed as it is not currently thought of as a significant mesendodermal inducer, and is linked to Wnt-based patterning influences.

An important aspect that has, so far, not been studied in depth is the level to which the expression of *Xatv/Lef1/4*, which is a direct target of Xnr signaling (Cheng et al., 2000; Tanegashima et al., 2000), is affected when *Xatv* translation is reduced. The level of *Xatv* expression was not changed at late blastula stage (stage 9.5; Supplemental Figs. 1A: i–l), but the dorsally disposed expression domain became greatly expanded during gastrulation, including a large animal poleward expansion (Fig. 3B; Supplemental Figs. 1A: m–t). The highly abnormal convergence/extension and involution movements in *Xatv* MO1 + MO2-injected embryos were associated with a failure to narrow *Xatv* expression to the dorsal midline (Fig. 3B: i, l). At late gastrula (stage 12.5), the expression domain remained widespread and extended more vegetally in *Xatv* MO1 + MO2-injected embryos (Fig. 3B: l). The observation that the *Xatv* expression domain is much broader than seen at any stage of normal control embryonic development indicates a true expansion of the expression domain rather than simply a MO-induced stabilization of *Xatv* mRNA. The impression of a dorsal lip margin (green arrowheads; Fig. 3B: l, m) was found to represent an indentation of the superficial cell layer, with the vegetal limit of *Xatv* expression (red arrowheads; Fig. 3B: m, n) representing the epiboly margin, and reflecting the lack of involution of the superficial region.

**Spatiotemporal regulation of Xnr signaling by Xatv is essential for organizer/mesendoderm formation and convergent extension**

The consequences of upregulated and maintained *Xnr* expression in *Xatv* MO1 + MO2-injected embryos during gastrulation were examined further by analyzing additional Xnr-responsive genes. The expression patterns of the trunk organizer markers *chordin* (*Chd*; Sasai et al., 1994) and *Antidorsalizing morphogenetic protein* (*ADMP*; Moos et al., 1995), and the prospective prechordal plate organizer marker *goosecoid* (*Gsc*; Cho et al., 1991) were initiated normally at the onset of gastrulation (data not shown), but were upregulated in *Xatv* MO1 + MO2-injected embryos at stage 10.5 (Figs. 4A, B; Supplemental Figs. 3A, C, G, K). In addition, the expression domains of these genes were greatly expanded into the animal/dorsal area from the mid-gastrula stage onward (Figs. 4A: k, B: h; Supplemental Figs. 3D, L). Consistent with the morphogenetic defects deduced from the abnormal *Xatv* expression pattern, there was no internalization or anterior-ward shifting of the *Gsc* and *Chd* expression domains (Fig. 4A: l; Supplemental Figs. 3D, L). In particular, *ADMP* expression remained, like *Xatv*, broad and non-internalized, with a morphological superficial indentation (green arrowheads; Figs. 4B: i, j) and vegetally extended epiboly margin on the dorsal side (red arrowheads; Figs. 4B: j, k). The head organizer marker *Cerberus* (*Cer*; Bouwmeester et al., 1996) was upregulated
and expanded by Xatv\textsuperscript{MO1 + MO2}-injection, although remaining dorsally disposed (Fig. 4C). In control embryos, a Cer-negative anterior midline domain corresponds to the anteriorly protruding (Gsc-expressing) prospective prechordal plate (Figs. 4C: a–f). In Xatv\textsuperscript{MO1 + MO2}-injected embryos, such a horseshoe-shaped Cer expression pattern was not observed. Substantial Cer expression was detectable in the subepithelial layer at the dorsal lip at stage 10.5, and Cer expression remained broadly expanded and adjacent to the epiboly margin over the next stages of gastrulation (Figs. 4C: g–i; data not shown). Because Cer is a downstream target of Nodal signaling, these observations are consistent with the idea that the relative balance between trunk and head tissues (Piccolo et al., 1999) is modulated by the action of Xatv on Xnr signaling.

Depleting Xatv function had a massive effect on the expression domains of Xbra (Xbra), a pan-mesodermal marker (Smith et al., 1991), and XWnt8, a ventral mesodermal marker (Christian et al., 1991; Smith and Harland, 1991). Unlike the previous reports (Branford and Yost, 2002; Tanegashima et al., 2004) and our results with single MO injections (Supplemental Figs. 1A: f, g). Xatv\textsuperscript{MO1 + MO2} together caused an enormous expansion of Xbra expression and, in most embryos, it covered almost the entire animal area, an effect that was maintained throughout gastrulation (Figs. 4D: g–l). In Lefty\textsuperscript{MO}-injected zebrafish embryos, the expression territory of the Xbra homolog no tail (ntl) expression was expanded by blastula (Agathon et al., 2001; Chen and Schier, 2002). In contrast, Xbra expression in late blastula stage Xatv\textsuperscript{MO1 + MO2}-injected embryos (as was seen for Xatv) was similar to control embryos (Supplemental Figs. 1: a–d), with expansion only beginning from the early gastrula stage (Fig. 4D; Supplemental Figs. 1A: e–h). Similarly, the expression of XWnt8 was intensified in the ventrolateral marginal zone at stage 10.5, while the arc of dorsal non-expression was increased in size (red arrowheads; Figs. 4E: a, e), complementing the expansion of organizer markers described above. At mid-gastrula stage, Xwnt8 expression covered the ventrolateral animal quadrant of Xatv\textsuperscript{MO1 + MO2}-injected embryos (Figs. 4E: h).

**Xatv depletion affects endodermal specification**

The modulation of induction processes by Xatv is also important in the specification of the normal endodermal territory. Previous reports failed to detect alterations in endodermin (edd) expression caused by Xlefty-MO injections (Branford and Yost, 2002). Gastrula stage edd expression marks axial mesoderm precursors as well as endoderm and, in the neurula, the notochord, prechordal plate, hatching gland, and entire endoderm, before becoming endoderm-specific at tailbud stages (Sasai et al., 1996). Below, we describe effects on edd expression that were detected at later stages in Xatv\textsuperscript{MO1 + MO2}-injected embryos (Figs. 6A: a–w). But, for this part of our gastrula stage analysis, we tried to select more rigorously endoderm-specific genes. We found that Xatv\textsuperscript{MO1 + MO2} injection not only expanded the expression domain of XSox17\textalpha, an Xnr-responsive pan-endodermal marker (Hudson et al., 1997; Osaka and Wright, 1999), but also increased the level of expression within this domain during gastrulation (Figs. 5A: a–f). Whole-mount in situ hybridization on bisected embryos showed that XSox17\textalpha expression was intensified and spatially expanded in the dorsal lip area (Figs. 5A: g, g’, h, h’). Dorsally, the expansion was dramatic in the superficial layer, while the ventral marginal zone showed expansion in both deep and superficial layers (Figs. 5A: g–h’). Another mesendodermal marker, expressed primarily in endoderm, Mixer (Henry and Melton, 1998), was also upregulated and expanded by Xatv\textsuperscript{MO1 + MO2} during gastrulation (Figs. 5B: a–f) to form a broad marginal zone band of expression around the entire embryo. This expression was located in dorsal and ventral regions of bisected embryos in the superficial (endodermal) and deep (mesendodermal) layers (Figs. 5B: g–h’).

We conclude that mesendoderm specification is initiated relatively normally in Xatv-depleted embryos, with dorsoventral patterning still evident, but that loss of Xatv function leads to massive expansion of both dorsal/ventral mesodermal and endodermal markers, a significant degree of global dorsalization of the embryo, and an associated failure of the involution and convergence/extension movements of gastrulation.

**Depletion of Xatv function expands mesodermal tissues during later embryogenesis**

Above, we showed the importance of Xatv as a primary negative feedback regulator of the strength, duration, and range of Xnr signaling at early embryogenesis. Previously, Branford and Yost (2002) concluded that post-gastrula Xlefty-deficient exogastrulae exhibit a reversal of the A/P axis but relatively normal patterning of the mesoderm and endoderm. We found, however, that the Xatv functional knockdown was translated into a substantial effect on cell fate allocation in later embryos. While the hyperdorsalization of the Xatv\textsuperscript{MO1 + MO2}-injected embryos results in substantial death during or after gastrulation if kept in the vitelline membrane, 80–90% survive if the membrane is removed, allowing phenotypic evaluation at later stages. Since Xatv\textsuperscript{MO}-induced exogastrulation made it difficult to directly compare the differences of marker expression and tissue formation between uninjected and Xatv\textsuperscript{MO1 + MO2}-injected embryos, we decided to compare them to classical exogastrulae induced by high salt (HS-exogastrulae) as a control.

The pan-neural marker, nrlp-1 (Richter et al., 1988; Knecht et al., 1995), was expressed in the ectoderm (blue line) but not in the dorsal midline of the mesendodermal mass (yellow line) in both types of exogastrulae (Figs. 6A: a–c). Similarly, the expression of cpl-1 and Xkrox20, markers for the dorsal forebrain and hindbrain, respectively (Knecht et al., 1995; Bradley et al., 1993), was detected in the expected pattern in the ectodermal part of the exogastrulae (data not shown). The expression of Xotx2 was significantly upregulated in the mesendodermal tip of Xatv\textsuperscript{MO1 + MO2}-induced exogastrulae (white arrowheads; Figs. 6A: d–f). Although Xotx2 is generally known as a marker for fore/midbrain at tailbud stage, prior to that it is expressed in the underlying prechordal mesendoderm, and is observed in the latter tissue in exogas-
trulae (Blitz and Cho, 1995; Pannese et al., 1995). We conclude that there was substantial expansion of the prechordal mesoderm fate in XatvMO1 + MO2-induced exogastrulae. The increased width and intensity of midline expression, and substantial ectopic expression in the anterior mesendoderm tip (Figs. 8A: g–i, red arrowheads) of Shh, a marker of prechordal plate, floor plate, and notochord (Ruiz i Altaba et al., 1995; Ekker et al., 1995), also supported a large expansion of prechordal plate in XatvMO1 + MO2-induced exogastrulae. The midline expression of Xatv in normal embryos at these stages (Fig. 6A: j) primarily marks the neural tube floorplate (blue arrowhead) and hypochord (yellow arrowhead), with weaker notochord expression (Cheng et al., 2000). In HS-exogastrulae (Fig. 6A: k), the surface location and punctate signal for Xatv in the dorsal mesendodermal mass (yellow arrowhead) indicated the formation of hypochordal tissue, and an ectodermal Xatv signal was seen at the mesendodermal mass/ectoderm junction (blue arrowhead). Xatv expression in the dorsal mesendodermal mass was greatly upregulated and broader in XatvMO1 + MO2-induced exogastrulae (Fig. 6A: j–l), recognizable as increased hypochordal tissue on the upper surface of the sections in Figs. 6B). The relative area of this (hypochord + notochord) tissue in transverse sections of XatvMO1 + MO2-induced exogastrulae (Figs. 6B: a–d) was more than two times larger than in HS-exogastrulae (Fig. 6B: e). Similarly, the expression of MyoD, a somitic mesoderm marker (Hopwood et al., 1989), was elevated and expanded (Figs. 6A: m–r). Quantitation of the total amount of mesodermal tissue, including somite and ventral mesoderm, in typical cross-sections from multiple embryos showed that it was approximately two-fold greater in XatvMO1 + MO2-induced ones compared to HS-induced ones (green dashed lines; Figs. 6B: a–d, f). In addition, histological analysis showed, within the mesodermal tissue in XatvMO1 + MO2-induced exogastrulae, a stack of layers of elongated cells with lozenge-shaped nuclei, indicative of differentiated somitic muscle (yellow dashed line; Fig. 6B: d); this tissue demarcation was less obvious in HS-exogastrulae. This somitic domain was determined from the residual MyoD signal on sections of embryos subjected to whole-mount in situ hybridization. Global endoderm formation between the two types of exogastrulae as marked by expression of endodermin (edd; Sasai et al., 1996) was similar in the two types of exogastrulae, being detected in the entire protruded mesendoderm (Figs. 6A: t–w). The XatvMO1 + MO2-induced exogastrulae, however, showed broad and strong edd staining in the midline, corresponding to the broad Shh-positive domain (yellow arrowheads; Fig. 6A: w). edd normally marks

Fig. 5. Xatv is required for normal endoderm fate specification. (A) In situ hybridization with Sox17α in uninjected and XatvMO1 + MO2-injected embryos during gastrulation. (a–c) Uninjected embryos. (d–f) XatvMO1 + MO2-injected embryos. (a–d) Vegetal view, dorsal upwards. [Quantitative analysis of alterations shown: d, n = 6/6.] (e, f) Lateral view. [Quantitation: c, n = 8/8; f, n = 9/10.] (g, h) Sox17α expression at St. 10.5 detected by in situ hybridization after bisection through the center of the dorsal lip of (g) uninjected (n = 10/10) and (h) XatvMO1 + MO2-injected (n = 9/10) embryos (dorsal to the left). (g′, h′) Magnified views, dorsal side of panels g and h, compared to (g″, h″) magnified ventral side views. (B) Mixer expression in (a–c) uninjected and (d–f) XatvMO1 + MO2-injected embryos during gastrulation. (a–e) Vegetal view, dorsal to the top. [Quantitation: d, n = 6/6; e, n = 8/8.] (f) Lateral view (n = 8/8). (g, h) Mixer expression at St. 10.5 detected by in situ hybridization after bisection through the center of the dorsal lip of (g) uninjected (n = 8/8) and (h) XatvMO1 + MO2-injected (n = 10/10) embryos (dorsal to the left). (g′, h′) Magnified views, dorsal side of g and h compared to (g″, h″) magnified ventral views. Green arrowheads: dorsal lip. Red arrowheads: most animal/ anterior expression limit of Sox17α (A) and Mixer (B). White brackets: separation of blastocoel floor from the edge of Sox17α (A) and Mixer (B) expression domains in the superficial layer. Black brackets: breadth of Mixer-negative area in the dorsal mesoderm region (B: g′, h′).
the developing notochord in late neurula embryos (Sasai et al., 1996) and our analysis showed only weak edd expression in the notochord in both uninjected embryos and HS-exogastulae at stage 25 (Figs. 6A: s, t, v). Therefore, it is likely that the axial-type edd expression detected in the Xatv-deficient exogastulae reflects the increased notochord, with the failure to downregulate edd expression perhaps reflecting a delayed differentiation process. Overall, the elevated Xnr signaling during gastrulation in Xatv-deficient embryos leads to an increased allocation of cells towards prechordal mesoderm, notochord, hypochord, and other mesoderm fates such as somite and ventral mesoderm at later stages.
Increased range of Xnr signaling induces the expansion of Xbra expression

In XatvMO1 + MO2-injected embryos, the highly upregulated and expanded expression of Xnr-responsive genes, such as Xbra and Xatv itself, in the presence of intensified but only slightly expanded Xnr1 and Xnr2 expression suggests that Xnr ligands are inherently capable of long-range signaling in the very large X. laevis embryo. To test this hypothesis, we examined whether the expansions of the expression territories of the Nodal-responsive genes were blocked by inhibitors of Nodal signaling: the secreted Xnr-specific inhibitor, CerS, which directly binds Xnrs and inhibits signaling non-cell autonomously (Piccolo et al., 1999; Agius et al., 2000), and tALK4, a dominant-negative type I receptor that cell autonomously inhibits signaling by Nodal, Xnr1, Xnr2, Xnr4, and Activin (Reissmann et al., 2001). XatvMO1 + MO2 were injected into 1-cell stage embryos, which were then injected at the 32- to 64-cell stage in a single A- or B-tier blastomere (Moody, 1987) with CerS or tALK4 RNA mixed with β-galactosidase (β-gal) RNA as lineage tracer. While the injection of β-gal RNA alone did not suppress the ectopic Xbra expression (Figs. 7B: a–c; n = 8/8), CerS produced from the labeled clone of cells gave rise to a marked patch of non-Xbra-expressing cells (Figs. 7B: d–f; n = 9/10). Because CerS is secreted, ectopic Xbra expression in XatvMO1 + MO2-injected embryos was inhibited in cells both within and away from the clone. When the injected clone was distributed to overlap the marginal region, tALK4 effectively blocked the endogenous Xbra expression domain in control embryos (Figs. 7A: d, d'; n = 7/7). The cell-autonomous tALK4 inhibitor blocked ectopic

Fig. 7. Evidence for the expansion of Xbra expression by long-range Xnr signaling after Xatv knockdown. (A) In situ hybridization with Xbra on embryos at St. 10.5. (a) Uninjected embryo. (b) XatvMO1 + MO2-injected embryo. (c) CerS-injected embryos. (d) tALK4-injected embryo. (d') High magnified view of panel d. (a–d) Lateral views. Red-gal staining in panels c and d detects the descendants of the cell injected with CerS and tALK4, respectively. (B) Xnr-specific inhibitors prevent ectopic Xbra expression in Xatv-deficient embryos. In situ hybridization on embryos at St. 10.5 detects Xbra expression, with red-gal staining detecting the descendants of the cell injected with RNA encoding the inhibitor (see text for detailed experimental design). Injection of β-gal (250 pg) does not affect ectopic Xbra expression (a–b'). CerS (500 pg) inhibits ectopic Xbra expression non-cell autonomously (d–e'), whereas tALK4 (500 pg) suppresses the ectopic Xbra expression cell autonomously (g–h'). (c, f, i) Simplified diagrams showing the effects of ectopic Xbra expression by β-gal, CerS, and tALK4, respectively. Red dots represent the clone of cells that express β-gal, CerS, and tALK4, respectively. "−" symbols in panels f and i show inhibition of Xnr signaling non-cell and cell autonomously by CerS and tALK4, respectively. (a, e) Animal views. (b, d, g, h) Lateral views skewed ~45° animal-ward. (d', e', g', h') Magnified views of the yellow bracketed area of panels a, b, d, e, g, and h, respectively. Green arrowheads in panels A and B indicate animal pole. (C) In situ hybridization with Der in (a, b) un.injected and (c, d) XatvMO1 + MO2-injected embryos. Dorsal views, with (d) angled downward slightly to visualize the animal-ward extent of Der signal.
Xbra expression only in lineage-labeled cells in XatvMO1 + MO2-injected embryos (Figs. 7B: g–i; n = 7/8). These data strongly suggest that the expanded Xbra (and other marker) expression in XatvMO1 + MO2-injected embryos was a direct result of an increased range of Xnr signaling associated with the intensified but only slightly expanded Xnr1/2 expression domain.

Recent studies with overexpression and cleavage mutants of the TGFβ-related molecule Derrière (Der) have demonstrated transcriptional feedback loops and heterodimeric interactions between Xns and Der that may regulate mesoderm specification and patterning (Sun et al., 1999; Takahashi et al., 2000; Lee et al., 2001; Onuma et al., 2002; Eimon and Harland, 2002). We therefore examined how Xatv knockdown influences Der expression. The expression pattern of Der showed no noticeable alteration in XatvMO1 + MO2-injected embryos at stage 10.5, when Xbra expression has already expanded massively animal-wards (Fig. 7C: c; n = 21/26; Figs. 4D: g, j). At stage 11.5, however, Der expression became significantly expanded into the dorsal animal quadrant (Fig. 7C: d; n = 13/17). Because the expanded Der expression occurs after that of Xbra, we conclude that the dramatic expansion of Xbra expression in Xatv-deficient embryos is highly associated with elevated expression of Xnrs and the expansion of Xnr signaling, although there could be some interaction with Der (see Discussion).

Xatv and Xbra synergistically affect Xnr expression

The limited expansion of Xnr2 expression in XatvMO1 + MO2-injected embryos, in the presence of a much broader response of other Xnr-responsive genes, suggested the existence of a remaining inhibitory influence blocking the Xnr auto regulatory loop. Xbra and Xnr2 are normally expressed in mutually exclusive domains encircling the marginal zone of gastrulation stage embryos (Kumano and Smith, 2000; Eimon and Harland, 2002). Kumano et al. (2001) found that overexpressing Xbra...
reduced Xnr2 expression in the ventral marginal region, and that production of a dominant negative Xbra, Xbra-EnR (Conlon et al., 1996), expanded Xnr2 expression in the same region. Because Xbra is a transcriptional activator, a simple model for the XatvMO1 + MO2-injected embryos is that the broad expansion of Xbra results in the induction of a factor that then represses Xnr transcription. Consistent with this idea, we found that the use of Xbra-EnR to block Xbra function in XatvMO1 + MO2-injected embryos led to a substantial expansion of the Xnr expression territory, much larger than in embryos receiving either Xbra-EnR or XatvMO1 + MO2 alone. Xbra-EnR injection into control embryos prevented endogenous Xbra expression in both superficial and deep cells (Fig. 8B; n = 11/11; Supplemental Figs. 4B, B', G, G'; data not shown), and produced a slight animal-ward expansion of superficial Xnr2 expression during gastrulation (Figs. 8D, D'; n = 15/16; Supplemental Figs. 4D, D', I, I'; also see Kumano et al., 2001). Notably, the intensity of the Xnr signal in the expanded Xnr2 expression domain was increased in the β-gal-marked Xbra-EnR-expressing cells compared to un.injected or XatvMO1 + MO2-injected embryos (Figs. 8A–D'). This difference was more evident when the intensity of Xnr2 expression was compared without labeling the injected clone by β-gal expression, in which case Xnr2 expression was detected as a punctate perinuclear signal (Supplemental Fig. 4). Production of Xbra-EnR in XatvMO1 + MO2-injected embryos expanded the region that showed this higher signal intensity even farther animal-ward during gastrulation (Figs. 8E, E'; n = 28/31; Supplemental Figs. 4E, E', J, J'). Some embryos receiving Xbra-EnR RNA, with or without previous XatvMO1 + MO2 injection, showed strong Xnr2 expression in cells laterally adjacent to β-gal-marked cells (data not shown). This non-autonomous effect plausibly arises via increased Xnr secretion from Xbra-EnR-producing cells leading to the stimulation of Xnr expression in those adjacent cells.

An interesting observation here, however, was that despite the ability of the Xbra-EnR to work in deep and superficial cells as described above, the expanded Xnr2 expression in all cases remained in the superficial cell layer, which is same layer selectivity shown by normal endogenous Xnr2 expression (Figs. 8C', D', E'; Jones et al., 1995; Eimon and Harland, 2002). Collectively, these data suggest that Xbra expression acts as a potent strong indirect transcriptional influence on Xnr autoregulation, effectively blocking the expansion of Xnr transcription, but not the spreading of Xnr signals, in Xatv-deficient embryos. Xbra and Xatv therefore synergistically regulate Xnr autoregulation at the transcriptional and extracellular levels, respectively (Fig. 8F).

Discussion

We have produced evidence that the expression and range of action of Xnr in the Xenopus embryo are tightly regulated by Xatv-mediated blocking of the Xnr autoregulatory loop, and that the transient and restricted nature of expression is further assured by indirect Xbra-mediated transcriptional repression. These data are integrated into a model shown in Fig. 8F. In the absence of these negative regulatory influences, the induction of target genes of Xnr/activin-like signaling becomes massively expanded, in some cases (represented by Xbra, for example) being able to cover the entire animal cap. Such a huge expansion was not appreciated from the previous publications on Xatv interference, and likely reflects our concurrent targeting of both Xatv alloalleles via splicing and translation-blocking MOs, as discussed below. An interesting feature currently under study is the specific competence of the superficial layer to activate Xnr transcription, which was found both in normal embryos and in those in which the function of both Xatv and Xbra was reduced. Our findings extend significantly the previous findings on the knockdown of Xatv function in Xenopus, and increase our appreciation of the interaction and potency of extracellular Xatv and intracellular Xbra as regulators of Xnr induction during fate specification in Xenopus. At a general level, our results emphasize the degree to which, in all vertebrate embryos, the Nodal signaling pathway is under several layers of restrictive influence in order to limit its range in embryonic tissue, which can otherwise induce fate alterations very far from the ligand source.

Synergistic inhibition of Xatv function by Xatv4- and Xatv6-specific MOs

Recent studies injecting either of two different translation-blocking Xlefty/Xatv morpholinos concluded that Xlefty/Xatv spatially limits Nodal target gene expression, but in a fairly restricted fashion around the dorsal organizer domain (Branford and Yost, 2002; Tanegashima et al., 2004). The less dramatic effects compared to our findings may be related to targeting only Xatv4 or Xatv6. Our translation-blocking XatvMO1, which matches 18 nucleotides of Xatv6, did not inhibit Xatv6 mRNA translation in vitro (Fig. 1). Similarly, the XleftyA- or XleftyB-specific MOs of Branford and Yost (2002) had uninterrupted A-to-B, or B-to-A, cross-matches of only 11 and 16 nucleotides, respectively, thus reducing the likelihood of cross-copy knockdown. No results from coinjecting both of their MOs were reported. A general finding from our studies was that single MO injections were less effective at reducing Xatv function than mixtures of lower doses of MO that target Xatv4 and Xatv6 simultaneously (Fig. 2). The coinjection of splice blocker XatvMO2 and translation blocker LeftyB-MO, which both target Xatv6, was also less effective than simultaneously targeting Xatv4/Xatv6 (Fig. 2A; Supplemental Fig. 1B). Overall, we conclude that more reproducible and stronger knockdown of Xatv function is achieved by synergistically inhibiting both Xatv4 and Xatv6 function.

Xatv and the strength and duration of Xnr signaling during gastrulation

Our analysis of Xatv-deficient embryos over several blastula/gastrula stages suggests a difference in the timing of negative feedback regulation by Lefty/Antivin on Nodal signaling between zebrafish and Xenopus embryos. In zebrafish, the upregulated and expanded expression of the Xbra homolog ntl was already present during blastula stages
Because mesendodermal specification and patterning involve an integrated response to many different signals, including Xnr, Der, Wnts, BMPs—all having overlapping, dynamic, and interdependent spatiotemporal expression and functional characteristics that are still far from well understood—it is difficult to define how much the transcriptional response within embryonic tissues to reduced Xatv function is attributable only to specific inducers, i.e., the Nodal-related factors, or to which ones within this family. In other words, a key issue in these and other studies (Agius et al., 2000; Lee et al., 2001; Eimon and Harland, 2002; Howell et al., 2002) is how much the expanded expression of Xbra and additional Xnr target genes reflects increased Xnr signaling alone, or incorporates effects from inducers such as Der, which has a delayed but substantial expansion in XatvMO1 + MO2-injected embryos (Fig. 7C). Der may be particularly relevant here. It has been suggested to act as a relay inducer that maintains mesendoderm induction during late gastrulation/tailbud stages, principally to produce the posterior mesendoderm (Sun et al., 1999; White et al., 2002). Significant cross-activation between Xnrs and Der in overexpression assays (Takahashi et al., 2000; Lee et al., 2001; Onuma et al., 2002; Eimon and Harland, 2002), and the possibility of promiscuous ligand interactions (e.g., Osada and Wright, 1999; Yeo and Whitman, 2001; Eimon and Harland, 2002) complicate the dissection of inductive events from Xnr and Der in normal and Xatv knockdown situations. It is possible, for example, that the primary functional inducer in vivo is an Xnr–Der heterodimer, although it is also possible that such dimmers only form in the overexpression assays used so far.

An additional issue is the true specificity of CerS, which is currently thought to be Xnr-specific, but has been shown to inhibit the induction of Der, Xbra, and Xnr1 expression by exogenous Der in animal cap assays (Eimon and Harland, 2002). The potential for significant cross-induction, however, makes it plausible that CerS is indeed an Xnr-specific inhibitor that does not physically interact with Der. We currently consider the early-expanded expression of target genes such as Xbra, Xatv, Xwnt8, and several organizer markers, to represent a response to signaling from Xnr and not Der. We base this conclusion upon: (1) Xnr and Der activate the formation of distinct transcriptional regulatory complexes, Fast1-containing ARF1 or Fast3-containing ARF2, respectively, at early and late gastrula stages. ARF1 and ARF2 have been independently linked to the different timing with which maximal Smad2 phosphorylation is induced by overexpressed Xnr (stage 9) or Der (stages 10–10.5: Lee et al., 2001; Howell et al., 2002). (2) Overexpression of Xnr1 or Xnr2 inhibits expression of Fast3, which normally begins during gastrulation (stages 10.25–11: Howell et al., 2002). The latter finding implies that extending the activity profile of Xnr signaling after Xatv knock-down (e.g., Figs. 3A: m) could shift Fast3 expression even later, to mid-gastrulation, thereby minimizing the involvement of Der/ARF2 transcriptional responses in the early stage expansion of the mesendodermal territories. The Xnr–Der heterodimerization mentioned above, however, opens the possibility that Der may contribute to the expanded

(Xagathon et al., 2001; Chen and Schier, 2002). In Xenopus, in contrast, Xbra and Xatv expression only became significantly different between control and XatvMO1 + MO2-injected embryos during mid-gastrula stage, concurrent with the dysregulation in Xnr expression (Figs. 3B, 4D; Supplemental Fig. 1A). Related to this observation, there are differences in normal frog and fish embryos in the spatiotemporal expression of Lefty/antivin expression. In zebrafish, lefty/antivin expression already encircles the margin during blastula stages (Thiese and Thiese, 1999; Biggrove et al., 1999), while, in Xenopus, marginal zone Xatv expression is first robustly detected just at or prior to the onset of gastrulation (Cheng et al., 2000; Tanegashima et al., 2000). While the relevance of this temporal difference to the mechanisms of embryonic patterning in each species is not known, a conserved feature in both fish and frog embryos is that Lefty/Antivin-mediated negative feedback is essential during gastrulation for determining the appropriate level of Nodal signaling associated with cell fate specification and the differentiation of mesendodermal tissues at late embryogenesis (Fig. 6; Agathon et al., 2001).

Xatv and long-range signaling by Xnrs

Reduced Xatv function greatly expands the expression during gastrulation of markers of the organizer (Gsc, Chd, ADMP, Cer), mesoderm (Xbra, XWnt8), and endoderm (Mixer, XSox17), as well as that of Xatv itself (Figs. 3B, 4, 5; Supplemental Fig. 3). Collectively, our results are consistent with the idea that the previous Xnr knockdown studies that germ layer specification and patterning require careful modulation of the extent of Xnr signaling. In normal embryos, Xnr signaling could occur via direct diffusion/transport of the ligand through embryonic tissue, although previous evidence argues both for and against Xnr being long-range signals (Jones et al., 1996; White et al., 2002; Williams et al., 2004), or depend upon Xnr–Xnr autoactivation. The observation that Xnr1 and Xnr2 expression remains localized to the marginal zone, together with gross animal-ward expanded expression of target genes in Xatv-knockdown situations, is consistent with induction by long-range ligand signaling, as proposed for Sqt in zebrafish and Xnr2 in Xenopus (Chen and Schier, 2001; Williams et al., 2004). That the target genes are induced as a direct result of increased Xnr signaling is supported by the ability of the cell non-autonomous CerS and cell autonomous tALK4 Xnr-inhibitors to block the ectopic Xbra expression induced by XatvMO1 + MO2-injection (Fig. 7B). For both inhibitors, the Xbra expression detected outside the lineage-labeled (inhibitor-expressing) clone and on the side farthest away from the marginal Xnr2 expression domain (Figs. 7B: d–i) fits with the concept that Xnr signaling is a very long-range influence in this large embryo. As compared to the direct diffusion of Xnr1 and Xnr2 from the equatorial region, it is also possible that a significant contribution towards the overall response of the embryo comes from the increased expression of the other mesoderm-inducing Xnrs, such as Xnr4, which is expressed in axial mesodermal tissue (Fig. 3m; Joseph and Melton, 1997).
expression of Xbra and other markers in Xatv-deficient embryos, even at St 10.5 when its expression level is similar to that in normal embryos (Fig. 7C). In addition, the Xnr–Der cross-inductive interactions raise the interesting question of why the apparently greatly increased Xnr signaling in Xatv-deficient embryos only increases Der expression during relatively late gastrulation stages, and why Der expression becomes broader only dorsally. More work is required to understand the interdependence of Xnr and Der expression and function in patterning. Nonetheless, the simplest inference is that the widespread ectopic expression of Xbra and other markers in early stage Xatv\(^{MO1 + MO2}\)-injected embryos is highly associated with an increased range of Xnr signaling, with maintenance of the ectopic gene expression domains and effects on tissue differentiation seen in later stage embryos resulting from cooperative induction by Xnrs and Der, whether they operate as homodimers or heterodimers.

**Xatv regulates morphogenesis indirectly**

Previous studies in zebrafish and mice showed that Lefty/antivin-deficiency enlarges the internalized mesoderm area in gastrula stage embryos (Meno et al., 1999; Feldman et al., 2002), as a result of excessive deep-cell internalization from an expanded germ ring and hypoblast (Feldman et al., 2002). The situation in Xenopus Xatv-deficient embryos seems somewhat different. The high level of Xnr signaling leads to substantial dorsalization and a failure to demarcate the future anterior–posterior zones properly with respect to each other, a situation incompatible with the production of concerted morphogenetic movements as defined by Ninomiya et al. (2004) (discussed in more detail below). In agreement with the data in Keller-type explant assays shown by Branford and Yost (2002), these defects lead to abrogated involution, mis-located convergence movements (Figs. 3B, 4A, B; Supplemental Figs. 3A–D), failure of blastopore closure, and exogastrulation. While our results basically agree with Branford and Yost (2002), we note significant differences between our embryos and theirs, which are perhaps also related to the greater reduction of Xatv function in our hands. For example, rather than remaining as distinct adjacent domains, we found extensive overlap of the expanded organizer (expressing Gsc, Chd, ADMP) and mesendoderm territories (both dorsal- and ventral-type; Figs. 4A, B, D; Supplemental Figs. 3E–L). The formation of a dorsal blastopore lip and subsequent blastopore groove in Xatv\(^{MO1 + MO2}\)-injected embryos implies the normal occurrence of the early gastrula-stage vegetal rotation that leads up to the first dorsal-side cell involutions (Winklbauer and Schürfeld, 1999). Normally, convergence forces in the marginal zone produce hoop stress around the blastopore that progressively closes the blastopore ventral-wards (Keller et al., 2000). Therefore, the failure to close the blastopore in Xatv\(^{MO1 + MO2}\)-injected embryos is probably directly related to the reduced cellular convergence in the marginal zone, associated with the long-lived, widespread, and overlapping expression domains of organizer markers.

Future work will address the detailed molecular and cell biological links between the large-scale repatterning of Xatv-deficient embryos and the misplaced convergence/extension movements. Xwnt11, the well-known regulator of the Wnt/planar cell polarity (Wnt/PCP) signaling pathway that is involved in convergence/extension, is a downstream target of Xbra (Heisenberg et al., 2000; Tada and Smith, 2000). Upregulated and shifted Xwnt11 activity in Xatv\(^{MO1 + MO2}\)-injected embryos could underlie the relocated morphogenetic movements if a broad supraphysiological level, rather than a normally graded amount, of Xwnt11 signaling in the marginal zone interferes with the generation of the vectorial information that underlies intercalatory tissue movements.

Linked to the latter concept is the idea that tissue movements caused by Wnt/PCP signaling are initiated in the vicinity of juxtaposed Chd and Xbra expression domains. Ninomiya et al. (2004) showed that convergent extension was initiated between conjugated animal caps that were previously treated separately with high or low activin doses. It also occurred in cell aggregates that had received a non-uniform activin signal ("graded explants"), in which counter-gradients of Chd and Xbra expression were established. In contrast, uniformly activin-treated explants with homogeneous Chd and Xbra expression did not undergo convergence/extension-based elongation. The latter condition mimics the overlapping Chd/Xbra expression that we observed at the dorsal marginal region of gastrulation-stage Xatv-deficient embryos (Fig. 4D; Supplemental Figs. 3C, D, J–L). The ideas of Ninomiya et al. (2004) allow an elaboration of the explanation offered by Branford and Yost (2002) for the exogastrulation of Xatv\(^{MO1 + MO2}\)-injected embryos: animal-ward relocation of the Chd–Xbra countergradient in Xatv knockdown embryos causes convergence extension movements to begin ectopically far above the dorsal marginal zone.

**Superficial vs. deep induction of Xnr2 expression: role of Xbra suppression**

Even when the inhibitory effects of Xatv and Xbra activity were blocked, the expanded Xnr2 expression remained in the most superficial cell layer—the same layer specificity seen in normal embryos (Figs. 8C’, D’, E’; Jones et al., 1995; Eimon and Harland, 2002). This layer-specific induction of Xnr2 expression is also obtained in animal caps that overexpress Xnr5 (S.T., M. Asashima and C.V.E.W.; submitted elsewhere), indicating a surprising level of control over the competence of the superficial vs. deep cells to respond to Nodal signaling. We consider this observation remarkable with respect to the traditional view of bilayered animal cap explants as comprising an inner sensorial-responsive layer and an outer cell layer that is refractory to induction. We are currently interested in characterizing the reason for this strict differential responsiveness, and note that Xnr3, which is primarily induced by Wnt signaling (McKendry et al., 1997; Hansen et al., 1997; Kofron et al., 2004), is also expressed in the superficial layer (Smith et al., 1995). That the expansion of expression in Xatv-deficient embryos of the endodermal marker Xsox17α occurs within the superficial (endoderm-fated) layer of the dorsal marginal zone (Figs. 5A: h, h’ may be linked to the observation that the
endodermal fate is specified by the highest levels of Nodal signaling, with these levels being reached in the cell layer expressing \( Xnr1/Xnr2 \).

There are two not necessarily mutually exclusive possibilities by which Xbra indirectly inhibits \( Xnr2 \) transcription, and helps to restrict \( Xnr2 \) expression to a narrow band of the superficial marginal zone. Both \( Xnr1 \) and \( Xnr2 \) are expressed in the blastopore-proximal region of the involuting marginal zone, supporting the notion of a primary role in inducing the formation of the head and/or anterior trunk. The narrowing of the marginal territory of \( Xnr1/Xnr2 \) expression at mid-gastrula compared to preceding stages (Jones et al., 1995; Eimon and Harland, 2002) likely reflects the beginning of the involution of the blastopore lip-proximal superficial layer. The broad equatorial band of \( Xbra \) expression, which results from early-stage \( Xnr \) signaling (Agius et al., 2000), is separated from the blastopore lip by a gap equivalent to the width of the \( Xnr2 \) expression domain (Kumano and Smith, 2000; Eimon and Harland, 2002). \( Xbra \) expression is strong in deep cells and much weaker in the superficial (future endoderm) layer. At the early gastrula stage, the \( Xbra \)-expressing cells approach the blastopore lip, and its expression level in the deep and superficial layers becomes more similar during mid-gastrulation (Vodicka and Gerhart, 1995; Eimon and Harland, 2002). Based on this architecture, Xbra could induce a suppressive signal from the deep cells that acts non-autonomously to inhibit \( Xnr2 \) transcription within the adjacent superficial layer. In addition, Xbra produced in the prospective endoderm, perhaps more effectively when its expression becomes increased in this layer, could induce a cell autonomous suppressor. The failure to spread \( Xnr2 \) expression inward to the deep marginal zone cells, while expanding significantly animalward, even when Xbra function is blocked in Xatv-deficient embryos, underscores the potential importance of the distinctive competence of the superficial layer to activate \( Xnr2 \) expression.

Other inducers, such as Der, which is expressed in deeper cells overlapping with \( Xbra \) expression, are not affected by these suppressive influences.

Acknowledgments

We thank Richard Harland, Hazel Sive, Daniel Kessler, William Smith, and Malcolm Moos for sharing reagents; Wright lab members for thoughtful comments on the experiments and the manuscript. We thank Yu Shyr for comments on statistical analysis. This work was supported by NIH grant number GM56238-08.

Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ydbio.2005.10.043.

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