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The lefty-related factor Xatv acts as a feedback inhibitor of Nodal signaling in mesoderm induction and L-R axis development in *Xenopus*

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SUMMARY

In mouse, *lefty* genes play critical roles in the left-right (L-R) axis determination pathway. Here, we characterize the *Xenopus* lefty-related factor antivin (Xatv). *Xatv* expression is first observed in the marginal zone early during gastrulation, later becoming restricted to axial tissues. During tailbud stages, axial expression resolves to the neural tube floorplate, hypochord, and (transiently) the notochord anlage, and is joined by dynamic expression in the left lateral plate mesoderm (LPM) and left dorsal endoderm.

An emerging paradigm in embryonic patterning is that secreted antagonists regulate the activity of intercellular signaling factors, thereby modulating cell fate specification. *Xatv* expression is rapidly induced by dorsoanterior-type mesoderm inducers such as activin or Xnr2. Xatv is not an inducer itself, but antagonizes both Xnr2 and activin. Together with its expression pattern, this suggests that Xatv functions during gastrulation in a negative feedback loop with Xnrs to affect the amount and/or character of mesoderm induced. Our data also provide insights into the way that lefty/nodal signals interact in the initiation of differential L-R morphogenesis. Right-sided misexpression of *Xnr1* (endogenously expressed in the left LPM) induces bilateral *Xatv* expression. Left-sided *Xatv* overexpression suppresses *Xnr1*/*XPitx2* expression in the left LPM, and leads to severely disturbed visceral asymmetry, suggesting that active 'left' signals are critical for L-R axis determination in frog embryos. We propose that the induction of *lefty*/*Xatv* in the left LPM by *nodal*/*Xnr1* provides an efficient self-regulating mechanism to downregulate *nodal*/*Xnr1* expression and ensure a transient 'left' signal within the embryo.

Key words: Nodal, lefty, Feedback inhibitor, Left-right axis, *Xenopus laevis*, antivin

INTRODUCTION

The vasculature and internal organs of vertebrates are arranged asymmetrically along the left-right (L-R) axis. In humans, deviations from the normal arrangement range from reversal of a few organs (situs ambiguus or heterotaxia), to reversal of all organs (situs inversus totalis). While the latter can be completely non-deleterious and remain undetected throughout life, other disturbances of asymmetry are often associated with complex cardiac defects, polysplenia, and venous malformations (Kosaki and Casey, 1998). The recent increase in our knowledge of the L-R axis determination process has come from two main directions.

First, a reverse genetic approach has led to the formulation of a basic network that regulates embryonic asymmetry, including intercellular signals such as Sonic hedgehog (Shh), nodal, lefty1 and lefty2, and transcription factors such as Pitx2 (for review, see Harvey, 1998). In chick, *Shh* expression is initially bilateral at Hensen's node, but becomes stronger on the left at later stages, and at specific stages in chick and mouse, *nodal* also shows left-biased expression at the node (Collignon et al., 1996; Levin et al., 1995; Lowe et al., 1996). A relay factor 'X' has been proposed to propagate node-proximate asymmetries through the paraxial mesoderm outward to the left lateral plate mesoderm (LPM), which develops extensive left side expression of *nodal* and, with a slight delay, *Pitx2*. A candidate for factor X is Caronte (Car), a Cerberus-related secreted BMP antagonist so far studied only in chick embryos (Rodriguez Esteban et al., 1999; Yokouchi et al., 1999; Zhu et al., 1999). Several features of *Car* (expression pattern, response to Shh, ability to induce *nodal* and randomize situs when misexpressed on the right side of the chick embryo), suggest that it relays node L-R asymmetries to the LPM. While the expression of *nodal* and *Pitx2* in the left LPM is conserved between vertebrates, the failure to find asymmetric *Shh* expression in mouse, *Xenopus*, and zebrafish highlights the need for further research into the regulation of L-R determination. In fact, very little is known about the very first events coordinating L-R axis specification in any vertebrate, although the asymmetric action of a L-R coordinator involving

the TGFβ-related Vg1 factor has been suggested in *Xenopus* (Hyatt and Yost, 1998).

Second, the genes disrupted in two classical mouse mutants exhibiting situs defects, *iv* (*inversus viscerum*) and *inv* (*inversion of turning*), were recently identified. The gene mutated in *iv*, in which approximately 50% of the homozygous mutants show inverted laterality (plus some heterotaxia), encodes a protein similar to axonemal dynein heavy chains (Supp et al., 1997). In contrast, homozygous *inv* mutants usually have complete situs reversal, and the corresponding gene encodes a large putative extracellular protein with multiple ankyrin repeats (Mochizuki et al., 1998). Although their function remains unknown, *iv* and *inv* act quite early in the L-R pathway, since the laterality of *nodal*, *Pitx2* and *lefty* expression is drastically affected in the homozygous mutants (Campione et al., 1999; Lowe et al., 1996; Meno et al., 1996; Piedra et al., 1998; Ryan et al., 1998).

Xenopus nodal and *Pitx2* homologs have been characterized (Campione et al., 1999; Jones et al., 1995; Joseph and Melton, 1997; Ryan et al., 1998; Smith et al., 1995). Of the four *Xenopus nodal-related* genes (*Xnrs*), only *Xnr1* is expressed asymmetrically during early tailbud stages (Lowe et al., 1996), like mouse *nodal*, and seems to be involved in L-R determination (Sampath et al., 1997). Left LPM expression of *XPitx2* begins later than *Xnr1* and continues on the left side of the heart and viscera during overt asymmetric morphogenesis. In addition, right-sided *Xnr1* misexpression induces *XPitx2* expression and leads to the randomization of organ situs. Together, these observations place *Xnr1* upstream of *XPitx2* and suggest that parts of the L-R determination cascade are conserved in *Xenopus*, chicken and mouse (Campione et al., 1999; Logan et al., 1998; Piedra et al., 1998; Ryan et al., 1998; Yoshioka et al., 1998).

Two highly diverged TGFβ-like genes, *lefty1*/*lefty2*, play vital roles in the L-R axis determination pathway in mouse. During early somitogenesis, *lefty1* and *lefty2* are expressed predominantly in the left neural tube floor plate and left LPM, respectively (Meno et al., 1997). Mouse embryos deficient for *lefty1* have a variety of laterality defects, most commonly thoracic left isomerism (Meno et al., 1998). Moreover, most mutant embryos show bilateral expression of *lefty2*, *nodal* and *Pitx2*, suggesting that midline *lefty1* expression maintains left and right side identities by somehow restricting the passage of 'left' signals to the right side.

Several genes have been identified in chick and mouse that show unilateral expression on the right side of the embryo, including *activin*, *cSnR*, *FGF8*, and *nkx3.2*, and interfering with these patterns can reverse situs (Boettger et al., 1999; Meyers and Martin, 1999; Patel et al., 1999; Schneider et al., 1999). In chick, it has been proposed that right-sided activin signaling leads to the asymmetric perinodal *Shh* signal by repressing *Shh* expression on the right side of the node (Levin et al., 1995). The expression of *FGF8* and *nkx3.2* on opposite sides in chick and mouse is perplexing and underscores our rudimentary understanding of L-R determination. Overall, while significant data suggest important interplay between the L and R side gene cascades, a central conserved feature in all vertebrates studied to date is the left-sided expression of *nodal*.

Based upon the recent characterization of the lefty-related factor antivin (atv) from zebrafish (Thisse and Thisse, 1999), we searched for similar genes in *Xenopus*. Zebrafish antivin, like mouse lefty, can act to suppress mesoderm formation in embryos, and shows asymmetric left-sided expression during early somitogenesis. But, because of difficulties in misexpressing *atv* on the left or right in zebrafish embryos, and the absence of *atv* mutants, the role of lefty-related factors in non-mammals has not been fully defined. Here, we address the function of *Xenopus antivin* (*Xatv*), a probable *lefty* ortholog. We propose that *Xatv* acts during two phases of embryogenesis: the specification of mesendodermal fates during gastrulation and the establishment of the L-R axis at early tailbud stages. First, *Xatv* expression is induced by, and can antagonize, strong mesoderm inducing ligands such as Xnr2 and activin. Its expression pattern during gastrulation identifies *Xatv* as a likely endogenous negative regulator of mesendoderm induction. At later stages, *Xatv* is expressed in the left LPM. Overexpression of *Xatv* on the left side of embryos suppresses left LPM expression of both *Xnr1* and *XPitx2*, and results in severe defects in internal organ morphogenesis. We suggest that an active 'left' signal, involving *Xnr1*/*nodal*, is required for L-R axis determination during amphibian embryogenesis, and that negative feedback through the induction of Xatv/lefty ensures that the signal initiating asymmetric morphogenesis is transient.

MATERIALS AND METHODS

Isolation of *Xenopus* antivin

Probing a *Xenopus* amplified dorsal lip library at low stringency with a 780 bp *Eco*47III-*Xho*I fragment of a zebrafish *antivin* cDNA (the ligand region and part of the 3′ UTR) yielded 53 overlapping *Xatv* cDNAs; the longest (#48) was sequenced on both strands using the Sequenase II kit (USB). A 1.8 kb open reading frame represents the entire protein coding sequence, with 32 bp and 662 bp of 5′ and 3′ untranslated sequence, respectively. The nucleotide sequence of *Xatv* was deposited in GenBank (accession number AF209744).

Embryo manipulations

In vitro fertilization, manipulation, and staging were as described by Kay and Peng (1991) and Nieuwkoop and Faber (1967). To misexpress *Xnr1* or *Xatv* but minimize their influence on mesendoderm induction/gastrulation, we injected plasmid DNAs (Sampath et al., 1997), from which expression is first activated at midblastula transition (pXEX) or early gastrulation (pCSKA). Four-cell embryos with dorsal/ventral pigmentation differences (Nieuwkoop and Faber, 1967) were injected on the right or left with CsCl-purified pXEX or pCSKA vectors alone, or containing either *Xatv* or *Xnr1*. Injections were approx. 60-70° (site 1) or approx. 120° (site 2) from the dorsal midline and 20° above the equator. We described previously that pCSKA/β-galactosidase injections into site 1 or 2 gave similar lineage tracing patterns: labeling of anterior/posterior LPM, in addition to other tissues, and generally not crossing the midline as in Sampath et al. (1997). Data from injections into either site were pooled (Tables 1-4). pCSKA/*Xatv* or pXEX/*Xatv* were constructed by inserting the *Xatv* cDNA into the *Eco*RV site of pCSKA (Condie et al., 1990) or *Sma*I site of pXEX (Johnson and Krieg, 1994). pCSKA/*Xnr1* and pXEX/*Xnr1* plasmids were described by Sampath et al. (1997). For scoring heart and gut looping at stage 43-45, embryos were fixed in MEMFA (Harland, 1991) and changed into methanol.

Animal cap assays

One-cell embryos were injected with RNA, animal caps explanted

(stage 8/9), cultured in 0.75× normal amphibian medium (NAM), collected at stage 10.5 or 24, and flash frozen in dry ice/ethanol for RT-PCR analysis.

RT-PCR

RT-PCR analysis on various embryonic stages or animal caps was as described by Chang et al. (1997). Primers were: *Xatv*, 5′ CGCCACTTCGATTTCCGTGTA 3′ and 5′ CGGGCTGGAGGAGC-TTTGACG 3′ (496 bp product); *FGFR* (Lemaire and Gurdon, 1994); *goosecoid* (*gsc*), *noggin*, *Xbrachyury (Xbra)*, *Xwnt-8*, Muscle-specific *actin* (Wilson and Melton, 1994); *Cerberus* (Bouwmeester et al., 1996); *EF1α* (Krieg et al., 1989); *endodermin* (*edd*) (Sasai et al., 1996); *globin* (Graff et al., 1994); and *chordin* (Sasai et al., 1995).

In situ hybridization

In situ hybridization was performed as described by Harland (1991) with modifications communicated by the author. Full-length antisense *Xatv* riboprobes were generated from *Bam*HI-linearized cDNA #48; sense riboprobes from *Asp*718-linearized cDNA #48. Another *Xatv* riboprobe corresponding to 441 bp of 3′ UTR (*Dra*I fragment from cDNA #48) was generated from *Pst*I-linearized plasmid, and gave the same expression data, with lower signal intensity as expected from the shorter probe. The data presented here likely represent expression of the A and B copies of *Xatv* from the pseudotetraploid *X. laevis* genome, both of which were isolated in our screen. Antisense *Xnr1* and *XPitx2* probes were described by Jones et al. (1995); Ryan et al. (1998). For double label analysis, fluorescein-labeled cardiac *troponin I* probe was synthesized from pXTnIc (Drysdale et al., 1994). Following in situ hybridization, embryos were postfixed in MEMFA and stored in methanol, or analyzed histologically. Embryos were photographed in methanol or after clearing in benzyl alcohol:benzyl benzoate (1:2 v/v).

Histological analysis

Embryos were dehydrated in an ethanol series, equilibrated to toluene:paraplast (Oxford Labware; 1:1 ratio) and paraplast embedded. Sections (10 μm) were counterstained with eosin (Sigma/Surgipath) or mounted directly.

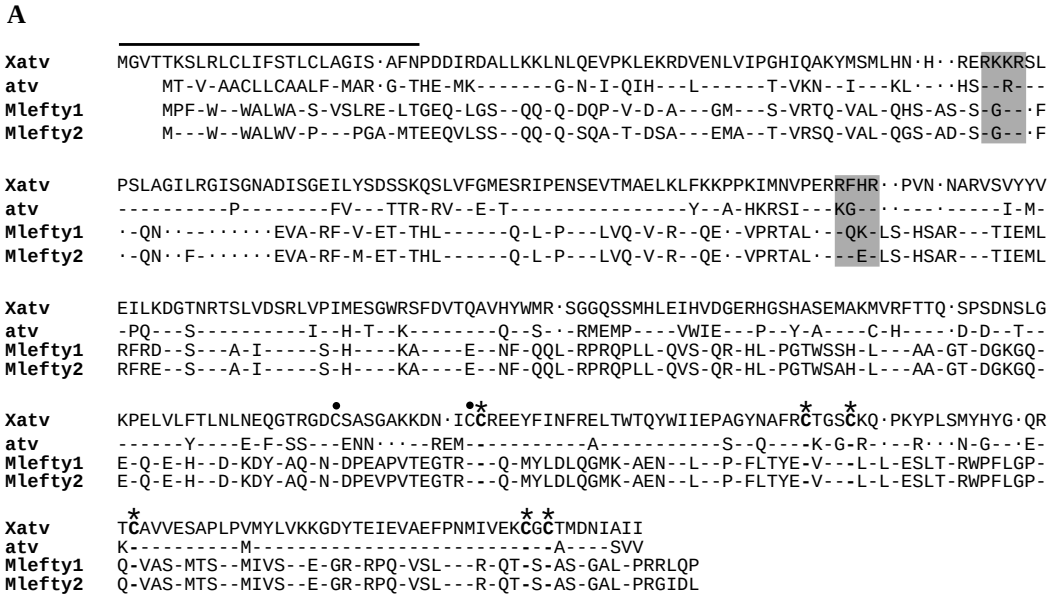

Fig. 1. Structure and expression of Xatv. (A) Xatv is 66%, 37% and 35% identical to zebrafish atv, mouse lefty-1 and lefty-2, respectively (overline indicates hydrophobic signal sequence; gray boxes, putative proteolytic cleavage sites; dashes, identical residues; dots, alignment gaps). Asterisks indicate cysteines of the TGFβ ligand 'cysteine knot'; additional cysteines residues conserved between lefty/atv factors are indicated. (B) TGFβ/BMP, lefty, and Xatv pre-proproteins. Gray, black and white boxes indicate signal sequence, pro region and mature ligand peptide, respectively. (C) RT-PCR analysis of *Xatv* expression during *Xenopus* embryogenesis (stages indicated; 0.1 embryo-equivalents/lane). FGFR, loading control; MBT, mid-blastula transition.

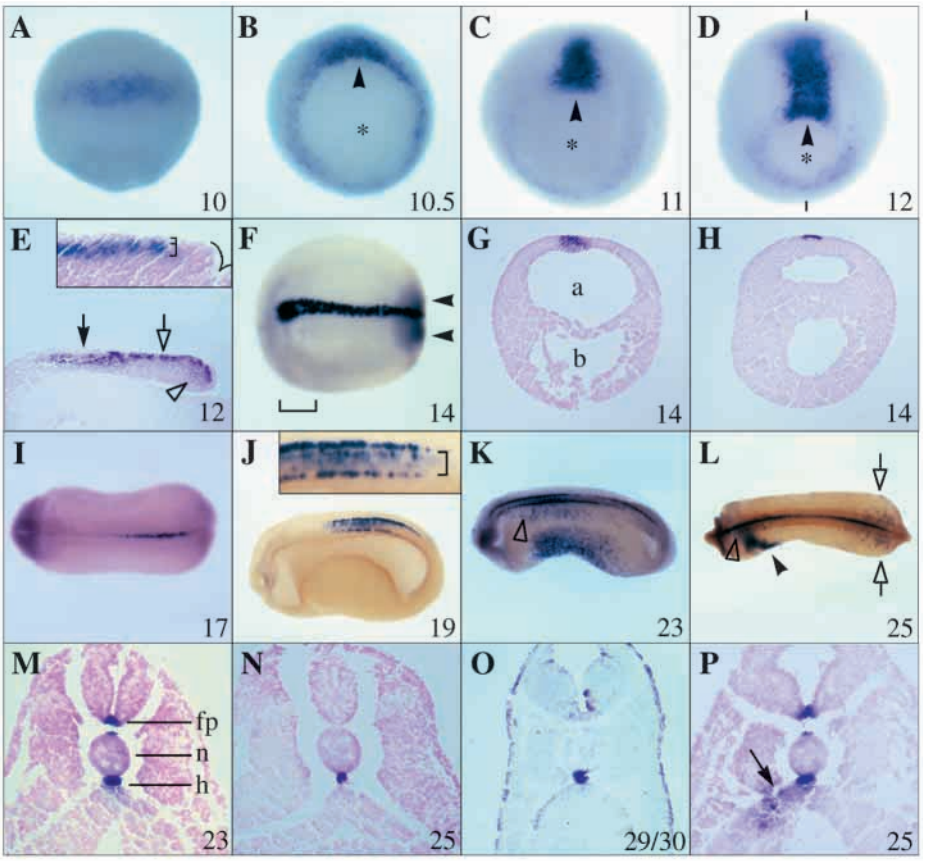

Fig. 2. Marginal zone and midline expression of *Xatv*. (A) Stage 10 embryo, dorsal view. *Xatv* transcripts are enriched dorsally in the marginal zone. (B-D) Stage 10.5, 11 and 12. Dorsovegetal views, dorsal up. *Xatv* expression within the forming dorsal midline. Arrowhead, dorsal lip; asterisk, yolk plug. (E) Stage 12, longitudinal section, approximate location shown in D. *Xatv* expression is seen in posterior neuroectoderm (open arrow), more anterior mesendoderm (solid arrow), and at lower levels in newly involuting mesendoderm (open arrowhead). Inset: higher magnification of posterior midline; *Xatv* is mostly expressed in deep neuroectoderm (bracket; dorsal lip indicated). (F) Stage 14. Midline expression, dorsal view, anterior left. Bracket shows the level of the section in G (arrowheads, circumblastoporal expression). (G) Stage 14, transverse section: *Xatv* expression in the prospective floorplate and underlying mesendoderm. (H) A more posterior section than in G shows *Xatv* expression only within the prospective floorplate. (I) Stage 17. Dorsal view, anterior left. (J) Stage 19. Dorsal view, anterior left. Inset: magnification of midline expression with transient notochord expression bracketed. (K,L) Stage 23 lateral view and stage 25 dorsal view, respectively (anterior left), showing *Xatv* expression in left dorsal endoderm (open arrowheads; see P for section), bilateral expression in the posterior dorsal endoderm (open arrows), and left LPM (solid arrowhead). (M) Stage 23. An anterior transverse section; *Xatv* expression in floorplate and hypochord. (N) Stage 25. (O) Stage 29/30. (P) Stage 25. Arrow, left dorsal endoderm expression. a, archenteron; b, blastocoel; fp, floorplate; n, notochord; h, hypochord.

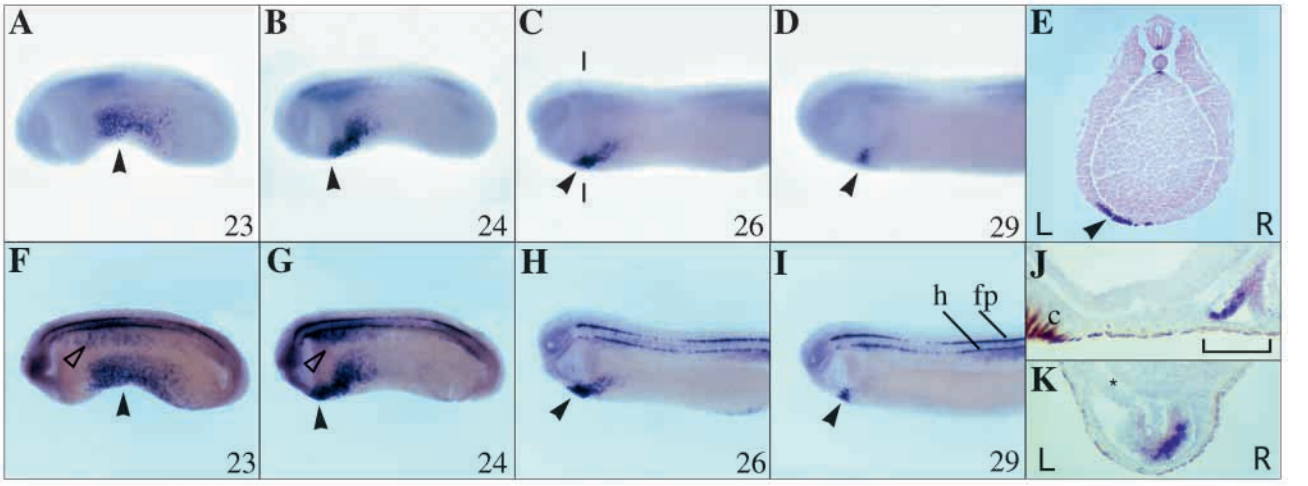

Fig. 3. Asymmetric *Xatv* expression. (A-D) Uncleared embryos, (F-I) cleared embryos (stages indicated); lateral views, anterior left. Solid arrowheads, *Xatv* expression in left LPM; open arrowheads, *Xatv* expression in left dorsal endoderm. (E) Transverse section of stage 25 embryo; approximate location indicated in C. Note expression in floorplate and hypochord. Solid arrowhead, *Xatv* expression in the left LPM. (J,K) Stage 32. Sagittal and transverse section, respectively, of heart regions of an embryo double labeled for *cardiac troponin I* (pink) and *Xatv* (purple), showing *Xatv* expression in the looping heart tube (bracket). *sectioning artifact. Epidermal coloration is normal embryo pigmentation. c, cement gland; fp, floorplate; h, hypochord.

RESULTS

Xenopus antivin

The 367 amino acid Xatv is 66% identical (81% similar) to zebrafish atv, and approx. 35% identical (approx. 54% similar) to mouse lefty1/lefty2 (Fig. 1A), and has the following features characteristic of lefty proteins. (1) It contains 6 of the 7 cysteines of the TGFβ superfamily 'cysteine knot', but lacks the fourth cysteine normally involved in covalent ligand dimerization (Fig. 1B, Kingsley, 1994). (2) It lacks the α helix between the third and fourth cysteines thought to promote dimerization through non-covalent interactions (Kingsley, 1994), suggesting that both Xatv and lefty function as monomers. (3) The carboxyl terminus of most TGFβ-related ligands terminates CX_1CX_1, while Xatv ends CX_1CX_8, more similar to lefty (CX_1CX_{13}; Meno et al., 1996). (4) Mouse lefty1 and lefty2 contain two putative pro-mature region proteolytic cleavage sites (RXXR), which may be used cell-specifically (Meno et al., 1997); similarly located sites exist in Xatv (Fig. 1B).

Xatv expression

RT-PCR analysis (Fig. 1C) detected *Xatv* expression shortly after the onset of zygotic transcription and at all later stages, with slightly higher levels in gastrula and early tailbud embryos. Transcripts of approx. 2.7 kb were detected by northern blot analysis of poly(A)$^+$ RNA from stages 11 and 18 (data not shown).

The spatial expression pattern of *Xatv* was determined by in situ hybridization analysis (Figs 2, 3). Expression is first observed just prior to the onset of gastrulation (stage 10) in the marginal zone, with a dorsal emphasis (Fig. 2A). At gastrulation, *Xatv* expression is concentrated at the dorsal lip (Fig. 2B) and, compared to the organizer marker *goosecoid*, is more superficial and proximate to the lip, and extends more laterally (data not shown). As gastrulation continues, the *Xatv* signal forms a crescent over an approx. 30° region of the dorsal marginal zone (Fig. 2C) and, at stage 12, marks the forming dorsal midline (Fig. 2D). Lower expression encircles the yolk plug from stages 10.5 to 12 (Fig. 2B-D). In the midline of the neurula (Fig. 2E), *Xatv* is expressed in the anterior mesendoderm, and posteriorly in the neuroectoderm; expression in both germ layers occurs in a transitional domain at mid-trunk level. Expression in the neuroectoderm is mostly restricted to the deep layer, although labeled cells are found in the superficial layer (Fig. 2E, inset). Lower expression is apparent in the newly involuting mesendoderm adjacent to the dorsal lip. In late neurula stage embryos (stage 14), *Xatv* is expressed predominantly in the dorsal axial midline (Fig. 2F). Anteriorly located sections show *Xatv* expression within the neuroectoderm and mesendoderm (Fig. 2G), while more posteriorly, *Xatv* is expressed in neuroectoderm only (Fig. 2H).

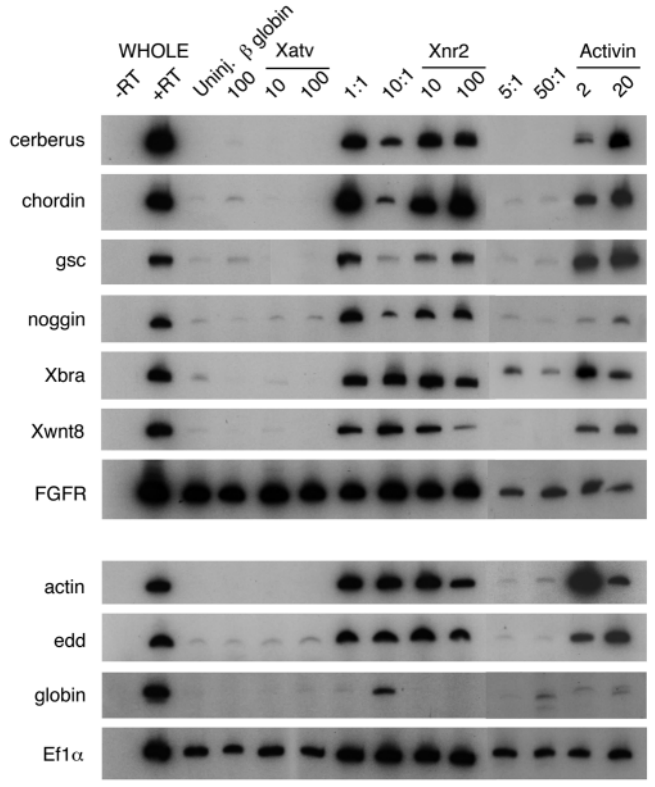

Fig. 4. Inhibition of Xnr2 and activin activity by Xatv. Animal caps injected with *Xatv* plus or minus *Xnr2* or *activin* RNA (pg/embryo indicated) were assayed at stage 10.5 or stage 24. At 1:1 ratios (10 pg each RNA), marker induction was similar to that caused by *Xnr2* alone. In contrast, a 10:1 mix of *Xatv*:*Xnr2* RNA suppressed organizer-specific markers (*cerberus*, *chordin*, *goosecoid* and *noggin*). At 5:1 or 50:1 ratios of *Xatv*:*activin* RNA, Xatv suppressed all markers tested.

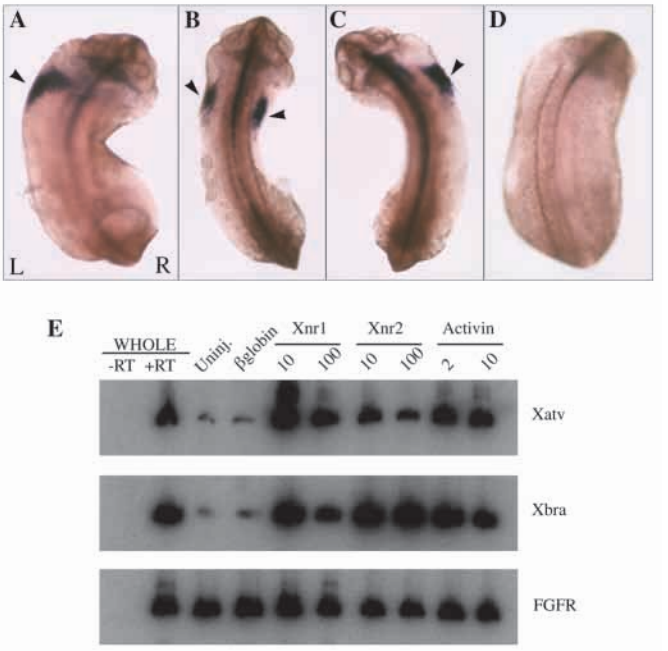

Fig. 5. Induction of *Xatv* in the LPM by Xnr1. pXEX/Xnr1 or pCSKA/Xnr1 was injected into the right side of 4-cell embryos to drive *Xnr1* expression from late blastula or gastrula stages, respectively, and *Xatv* expression analyzed at stage 24-25. Dorsal views, anterior up. (A) Normal asymmetric expression of *Xatv*; (B) bilateral expression; (C) right side expression and (D) no expression. Bilateral expression predominated (Table 1). (E) Animal caps injected with *Xnr1*, *Xnr2*, or *activin* RNA were assayed at stage 10.5 for *Xatv* and *Xbra*. *Xbra*, a pan-mesodermal marker, measured induction efficiency. FGFR, loading control.

Consistent with the earlier expression around the yolk plug, expression is maintained around the posterior blastopore (Fig. 2F). During tailbud stages, *Xatv* is transiently expressed in the notochord anlage (Fig. 2J), but over a more prolonged period there is robust expression in the floorplate and the hypochord, a structure thought to originate from dorsal endoderm (Fig. 2I,J,M-P). Indeed, at earlier stages, 2 or 3 dorsal-most endodermal cells underlying the notochord are labeled per section (data not shown). The floorplate/hypochord expression begins posteriorly (Fig. 2I), spreads anteriorly, and at later stages essentially extends throughout the A-P axis (Fig. 2K,L). In both structures, however, expression is somewhat discontinuous, particularly at the mid-trunk level (Fig. 3F-I). Thus, individual sections show labeling in hypochord or floorplate alone, or in both tissues (Fig. 2M-P). From approx. stages 22-25, *Xatv* is expressed in the left dorsal endoderm over the anterior half of the embryo (Fig. 2K,L,P) with an anterior limit in the foregut region (data not shown). Posteriorly, dorsal endodermal expression is bilateral (Fig. 2L).

Xatv displays transient expression in the left LPM (Fig. 3A-D,F-I), and is not found in the paraxial or intermediate mesoderm (Fig. 2M,N) – a pattern similar to that of *Xnr1* (Lowe et al., 1996). The LPM expression changes rapidly, initially appearing as a broad A-P band at the trunk level (stage 23; Fig. 3A,F), but becoming progressively restricted, anteriorly and ventrally, until it marks left ventral tissues within the heart precursor region (Fig. 3D,E,I). Sections of stage 28 embryos, when the myocardium is a simple epithelial sheet lying ventral to the just-formed endocardial tube, show that *Xatv* expression in the myocardium is not strictly unilateral, but is more extensive on the left than the right (data not shown). Later, *Xatv* is expressed on the right side of the definitive heart tube (Fig. 3K). In principle, the apparent left to right movement of expression within the cardiac tissue could arise from tissue movements and/or alterations in the expression domain. The precise relationship of *Xatv* expression with the L-R and A-P axes of the heart during the looping process is under investigation.

Xatv antagonizes Xnr2 and activin

Many TGFβ-like ligands can induce alterations in embryonic cell fate. Previously, chimeric BMP^{PRO}:$lefty^{MAT}$ proteins, which may function non-physiologically, were shown to neuralize animal cap ectoderm, suggesting that lefty ligands have anti-BMP activity (Meno et al., 1998). We tested for potential inductive properties of Xatv by injecting RNA into embryos and assaying gene expression in explanted animal caps. Markers for several tissue types were analyzed: dorsal endoderm (*cerberus*), pan-endoderm (*edd*), organizer-specific (*gsc*, *noggin*, *chordin*), pan-mesoderm (*Xbra*, muscle-specific *actin*), ventrolateral mesoderm (*Xwnt8*), and ventral mesoderm (*globin*). *Xatv* induced none of these markers (Fig. 4). Even high *Xatv* doses (1 ng/embryo) did not neuralize animal caps, as defined by the failure to induce *NCAM*/*Xotx2* and lack of effect on epidermal *keratin* expression (not shown).

To determine whether Xatv functions similarly to zebrafish atv, which antagonized mesoderm induction by activin in whole embryos (Thisse and Thisse, 1999), we coexpressed Xatv and a strong mesoderm inducer, Xnr2 (Fig. 4). At a 1:1 ratio (10 pg each RNA), marker induction was similar to *Xnr2* alone. In contrast, a 10:1 ratio (100 pg *Xatv*: 10 pg *Xnr2*), resulted in the suppression of organizer-specific markers (*gsc*, *noggin*, *chordin*, *cerberus*) while mesodermal markers (*Xbra*, *Xwnt8*, or muscle-specific *actin*) were relatively unaffected (Fig. 4). Notably, *globin*, a ventral mesoderm marker, was reproducibly induced by 10:1 *Xatv/Xnr2* RNA mixtures (Fig. 4). At higher ratios (500 pg *Xatv*:10 pg *Xnr2*), Xatv completely suppressed the induction of *gsc*, *noggin*, *chordin* and *cerberus*, while *Xbra* and *Xwnt8* decreased slightly (not shown). Xatv also antagonized the dose-dependent mesoderm inducer activin (Fig. 4). At a 1:1 ratio (2 pg *Xatv*:2 pg *activin* RNA), marker induction was similar to *activin* alone, although *globin* was induced by this combination once (data not shown). There was some variability in the response to mixtures of 5:1 or 50:1 of *Xatv*:*activin* RNAs (2 pg *activin* RNA used in each case), perhaps reflecting different responsiveness of animal caps from separate embryo batches. In two cases (5:1 or 50:1 mixtures), there was complete suppression of all general and dorsal mesendodermal markers tested (Fig. 4); while in one (a 5:1 *Xatv/activin* mixture) only the dorsal markers *chordin*, *cerberus* and *goosecoid* were completely suppressed (not shown). Although saturation loading of the translational machinery usually occurs at doses of several nanograms of RNA, we tested whether the suppressive effects of Xatv could be attributed to non-specific competition. Marker induction by a mixture of a translatable, irrelevant RNA (*β-globin*) with *Xnr2*, at doses up to 500 pg *β-globin*:10 pg *Xnr2*, was almost identical to that caused by 10 pg *Xnr2* alone (data not shown), indicating that Xatv specifically blocks nodal signaling pathways.

The potential for different translational efficiencies of these RNAs prevents conclusions on the amounts of active Xnr2, Xatv, or activin produced. Clarification of the stoichiometries of these antagonistic interactions may require purified functional proteins. Nonetheless, our data suggest that Xatv does not act as an inducer itself, but antagonizes potent mesoderm inducers such as Xnr2 and activin, thereby modulating cell fate specification during mesendodermal induction.

Xnr1 induces asymmetric *Xatv* expression

The unilateral expression during tailbud stages suggests that *Xatv* is involved in L-R axis determination. LPM expression of *Xnr1* begins (stage 18/19) earlier than *Xatv* (stage 23), potentially placing *Xatv* downstream of *Xnr1* in the L-R pathway. To test this hypothesis, we delivered plasmids encoding Xnr1 to either the left or right side of four-cell embryos and analyzed *Xatv* expression at stage 23-25 (see Fig. 3).

Embryos receiving left injections of *Xnr1* plasmids retained *Xatv* expression in the left LPM (Fig. 5A; Table 1). In contrast, the majority of embryos receiving right-sided *Xnr1* showed perturbed *Xatv* patterns, including expression bilaterally, or on the right only. The incidence of embryos bilaterally lacking *Xatv* expression was not altered significantly from controls (Fig. 5B-D; Table 1). A dose-dependent effect was seen (Table 1): at low doses (20 pg), most embryos retained left-sided *Xatv* expression, while 12% showed bilateral expression (Table 1). At higher doses (100 pg), most embryos had bilateral *Xatv* expression (55%; Table 1), and another 30% displayed right-side only expression. The induced ectopic expression pattern had mirror-image spatiotemporal characteristics of the

Table 1. Right sided *Xnr1* delivery perturbs asymmetric *Xatv* expression

Injections	Side inj.	*n* scored*	% left	% right	% bilateral	% neither
Uninjected	NA	65	88	-	1	11
Vector controls 100 pg	R	42	95	-	-	5
pXnr1 20 pg	R	66	79	6	12	3
	L	72	89	-	3	8
pXnr1 100 pg	R	33	12	30	55	3
	L	40	88	2	5	5

Embryos injected (doses indicated) with pXEX/Xnr1 or pCSKA/Xnr1 on the left or right were analyzed for *Xatv* expression in the left LPM at stage 23-25.

*****All surviving embryos were examined; this table includes only those with overall normal A-P and D-V axes (*n* scored; see Results text). Data were pooled from four experiments. -, not observed; neither, complete absence of expression. inj, injected; NA, not applicable.

endogenous left-side expression of *Xatv*, suggesting that right-sided *Xnr1* misexpression triggered a physiological left-side gene expression response. Consistent with previous reports (Sampath et al., 1997), section analysis showed that *Xnr1* did not induce ectopic tissues or secondary axes that might induce additional *Xatv* expression.

The inductive relationship between *Xnr1* and *Xatv* in tailbud stage LPM led us to test for a similar relationship during gastrulation. *Xatv* was robustly and rapidly induced in animal caps by *Xnr1*, *Xnr2*, or *activin* RNA (Fig. 5E), or activin protein (data not shown). Together with the overlapping spatiotemporal expression of *Xnr1*/*Xatv* during both stages of normal development, these data strongly suggest that *Xnr1* functions upstream of *Xatv* in both mesendodermal specification and L-R determination.

Xatv suppresses asymmetric gene expression

Right-sided *Xnr1* misexpression also induces *XPitx2* expression in tailbud stage LPM (Campione et al., 1999; Ryan et al., 1998). We explored the interactions between *Xnr1*, *XPitx2*, and *Xatv* by determining the effect of unilateral *Xatv* misexpression. Plasmids pCSKA/*Xatv* or pXEX/*Xatv* were injected into the right or left side of four-cell embryos, which were then analyzed for *XPitx2* expression at stage 24/25. Some embryos injected with 20 pg of *Xatv* plasmids on the left or right displayed an absence of left *XPitx2* expression, but at a low incidence, similar to controls (Fig. 6A,C; Table 2). However, the majority of embryos (69%) injected with 100 pg of *Xatv* plasmids on the left side lacked left *XPitx2* expression (Fig. 6B,D; Table 2). 50 pg doses (not shown) were either intermediate or similar to the 100 pg dose. The inhibition of asymmetric *XPitx2* expression was specific, since the bilateral expression of this gene in the head and cement gland was maintained (Fig. 6B,D).

Next, we asked whether *Xatv* suppressed *Xnr1* expression in the left LPM, reasoning that the absence of *XPitx2* in the LPM might have been caused indirectly if its putative upstream activator, *Xnr1*, was not expressed. Analysis of *Xatv*-injected embryos revealed an effect similar to that on *XPitx2*. Left or right-sided injections of low doses (20 pg), similar to the lack of effect on *XPitx2*, usually did not alter the left expression of *Xnr1* (Fig. 6E, Table 3). In contrast, left-sided injections (100 pg) suppressed left *Xnr1* expression in 74% of embryos (Fig. 6F, Table 3). Some embryos receiving higher *Xatv* doses (100 pg) on the right side also lacked both *Xnr1* and *XPitx2* expression (Tables 2, 3). Overall, the blocking of *Xnr1* and *XPitx2* expression in *Xatv*-injected embryos indicates that *Xatv* can function in vivo to negatively regulate *Xnr1* and *XPitx2* within the left LPM. Suppression of *Xnr1* expression by *Xatv*

Table 2. Left-sided *Xatv* overexpression suppresses asymmetric *XPitx2* expression

Injections	Side inj.	*n* inj.	*n* scored*	% left	% right	% bilateral	% neither
Uninjected	NA	NA	46	93	-	-	7
Vector controls 100 pg	R	170	119	94	-	-	6
	L	170	112	92	-	-	8
pXatv 20 pg	R	170	124	90	-	-	10
	L	170	126	90	-	-	10
pXatv 100 pg	R	170	124	81	-	-	19
	L	170	114	31	-	-	69‡

Embryos injected (doses indicated) with pXEX/Xatv or pCSKA/Xatv on the left or right were analyzed for *XPitx2* expression in the left LPM at stage 24/25 (data pooled from three experiments).

‡The alterations caused by *Xatv* injections are statistically significant compared to vector-injected controls ($P<0.001$, Fisher's exact test). See Table 1 for other notations and abbreviations.

Table 3. Left-sided *Xatv* overexpression suppresses asymmetric *Xnr1* expression

Injections	Side inj.	*n* inj.	*n* scored*	% left	% right	% bilateral	% neither
Uninjected	NA	NA	79	95	-	2.5	2.5
Vector controls 100 pg	R	79	76	97	-	1.5	1.5
	L	77	75	97	-	-	3
pXatv 20 pg	R	75	73	99	-	-	1
	L	78	73	92	-	1	7
pXatv 100 pg	R	86	83	78	-	-	22
	L	83	75	25	1	-	74‡

Embryos injected (doses indicated) with pXEX/Xatv or pCSKA/Xatv on the left or right were analyzed for *Xnr1* expression in left LPM at stage 21/22 (data pooled from four experiments).

‡Alterations caused by *Xatv* injections are statistically significant compared to vector-injected controls ($P<0.001$, Fisher's exact test). Other notations and abbreviations as in Table 1.

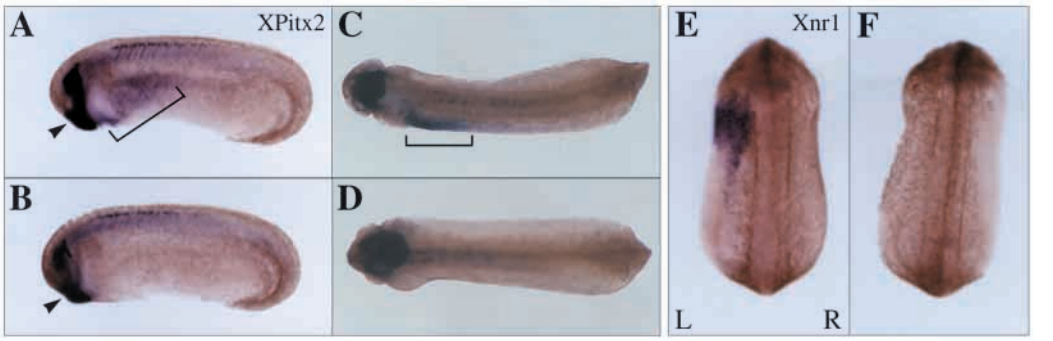

Fig. 6. Suppression of left LPM *Xnr1* and *XPitx2* expression by Xatv. pXEX/Xatv or pCSKA/Xatv was injected into the left side of 4-cell embryos and (A-D) *XPitx2*, or (E,F) *Xnr1* expression analyzed. (A,B) Lateral views and (C,D) dorsal views (anterior left). (A,C) Normal *XPitx2* expression in left LPM (bracket) is (B,D) suppressed by left-sided *Xatv* overexpression. Bilateral *XPitx2* expression in the eyes, head, branchial arches and cement gland (arrowheads in A,B) remains relatively unaffected. (E,F) Dorsal views, anterior up. (E) Normal *Xnr1* expression in left LPM is (F) suppressed by left-sided *Xatv* overexpression.

may account for the loss of *XPitx2* (see Fig. 8); other approaches will be needed to test whether Xatv blocks *XPitx2* expression directly.

Effect of *Xatv* misexpression on asymmetric organogenesis

As reported previously, right-sided misexpression of *Xnr1* (Sampath et al., 1997) and *XPitx2* (Campione et al., 1999; Ryan et al., 1998) can randomize the direction of heart and gut looping (e.g. Fig. 7C,D) when scored within a population of embryos. We assessed the consequences of *Xatv* misexpression on organ morphology and situs to correlate them with the molecular alterations in *Xnr1*/*XPitx2* expression described above. Embryos injected with pCSKA/*Xatv* or pXEX/*Xatv* on the left or right sides were cultured until stage 43-45 and scored for overall external/internal morphology, and situs of the heart and viscera (Fig. 7; Table 4). In these experiments, a small percentage of injected embryos failed to gastrulate, perhaps due to DNA toxicity; lower doses (20 pg) allowed greater survival than higher doses (100 pg; Table 4). In addition, higher survival occurred in embryos injected with pCSKA/*Xatv* than with pXEX/*Xatv* (Table 4), possibly reflecting a deleterious effect of earlier expression from pXEX (see Methods). Because defects in axial development affect L-R specification (e.g. Yost, 1998), embryos with abnormal A-P patterning (approx. 5% of injected embryos) were not analyzed for situs alterations. Of the remainder, 89% exhibited normal axial development (Fig. 7J), while 11% displayed ventral abnormalities (Fig. 7M), including edema and swelling. The latter may be associated with the defects in the visceral and cardiac systems described below.

Most embryos (63%; Table 4) injected on the left side with the highest *Xatv* dose (100 pg) developed indeterminate situs and/or dysmorphogenesis of both the heart and gut (54%), or gut alone (9%; Fig. 7J-O; Table 4). This incidence of visceral dysmorphogenesis (63%) is highly significant ($P<0.001$) compared to vector controls (16%; vector, 100 pg L) using Fisher's exact test, and agrees quite well with the 74% and 69% incidence of bilateral absence of *Xnr1* and *XPitx2*, respectively (Tables 2, 3). If the bilateral absence of *Xnr1*/*XPitx2* expression caused by left-sided *Xatv* misexpression were to cause global situs randomization, an approx. 35% incidence of combined heart/gut reversal would be predicted. In contrast, only a small fraction of left side-injected embryos developed situs reversals of both heart and gut (2/127; approx. 2%), heart alone (19/127; 15%), or gut alone (2/127; approx. 2%). Of the 19 with reversed hearts alone, gut situs was normal in seven, but indeterminate in the remaining twelve. Interestingly, a significant number of embryos (32%, compared to 20% in vector controls; $P<0.05$) receiving high doses of *Xatv* on the right also displayed visceral organ malformations (not shown) similar to those arising from left-sided injections. This result is consistent with the observation that some right-side injected embryos developed bilateral absence of *Xnr1* (22%) and *XPitx2* (19%) at tailbud stages (Tables 2, 3). One possibility is that the

Table 4. Morphological consequences of the absence of *Xnr1*/*XPitx2* asymmetric expression

					Heart reversed		Gut reversed			
Injections	Side inj.	*n* inj.	*n* scored*	Heart/gut normal	Gut normal	Gut indet.	Heart normal	Heart indet.	Heart/gut reversed	Heart/gut indet.
Uninjected	NA	NA	129	77	-	-	-	-	-	23
Vector controls 100 pg	R	233	170	79	-	-	1	-	-	20
	L	233	163	83	-	-	-	-	1	16
pXatv 20 pg	R	195	132	75	1	1	1	2	-	20
	L	197	123	73	2	-	-	-	-	25
pXatv 100 pg	R	210	137	60	3.5	1.5	-	2	1	32
	L	212	127	28	6	9	1.5	-	1.5	54‡

Embryos injected (doses indicated) with pXEX/Xatv or pCSKA/Xatv on the left or right were scored at stage 43-45 for heart and visceral orientation (data pooled from six experiments, presented as percentages).

‡Alterations caused by *Xatv* injections are statistically significant compared to vector-injected controls ($P<0.001$, Fisher's exact test). Notations as in Table 1 also apply. indet, indeterminate situs.

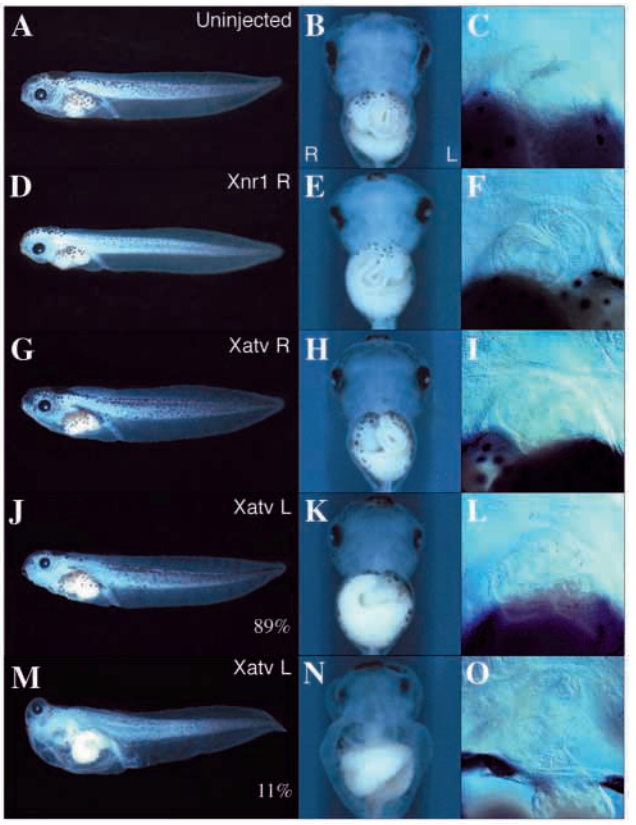

Fig. 7. Morphological analysis of *Xatv*-injected embryos. Plasmids (100 pg) encoding Xnr1 or Xatv were injected into either the right (R) or left (L) of 4-cell embryos and morphology analyzed at stage 43-45. (A,D,G,J,M) Lateral views; (B,E,H,K,N) corresponding ventral views; anterior up. (C,F,I,L,O) Higher magnification of heart region. (A-C) Uninjected embryo. (D-F) Reversed heart and gut situs in right-side *Xnr1* injections, for comparative purposes. (G-I) Normal heart and gut situs in right-side *Xatv*-injected embryos. (J-O) Two examples of embryos receiving *Xatv* on the left (percentage incidences indicated; see Table 4 for details).

injection of plasmids into 4-cell embryos, which are incompletely cleaved vegetally, could sometimes allow leakage to the left side. Such leakage was not, however, observed in pCSKA/β-galactosidase lineage tracing experiments (Sampath et al., 1997; not shown). Another possibility is that there is significant contralateral secretion of right side-overexpressed Xatv.

The different stages of analysis for *Xnr1* or *XPitx2* expression, or organ situs, preclude comparison of all three characteristics within the same embryos. To correlate as directly as possible the *Xatv*-mediated suppression of *Xnr1*/*XPitx2* expression with the situs abnormalities, we carried out experiments (not shown) in which one-third each of a batch of left-side *Xatv*-injected embryos were analyzed for *Xnr1*, or *XPitx2*, or allowed to develop further for situs scoring. In three experiments, the percentage of embryos bilaterally lacking *Xnr1* or *XPitx2* correlated well with the incidence of indeterminate situs/malformations of either the heart or gut, or both organs, and the overall results were completely consistent with the experiments in which these features were individually scored (Tables 2-4).

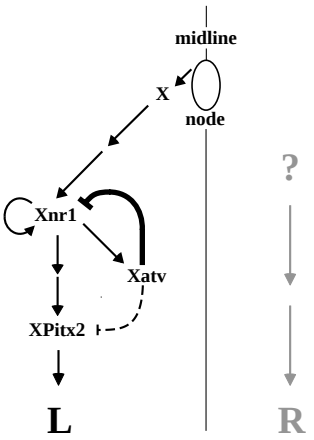

Fig. 8. A model for the role of Xatv in the establishment of L-R asymmetry. Time flows from top to bottom. A hypothetical factor 'X' that is proposed to propagate node-proximate asymmetries activates *Xnr1* within the left LPM during early tailbud stages. *Xnr1* activates *XPitx2* expression directly, or through intermediary steps. *Xnr1* also induces asymmetric *Xatv* expression. With a delay period, Xatv acts in a negative feedback loop to suppress *Xnr1* expression. Preliminary data suggest that *Xnr1* regulates its own expression. In the experiments reported here, *Xatv* may inhibit *XPitx2* directly (dashed bar), or perhaps more likely by directly suppressing *Xnr1* (solid bar). The possibility of yet undiscovered genes in *Xenopus* acting in a right-sided gene cascade, as in other species (see text), is indicated.

DISCUSSION

The complex spatiotemporal expression profile of *Xatv* during embryogenesis indicates a role in several developmental processes. Our functional data imply that Xatv acts as a feedback antagonist of Nodal signaling in two of these: mesendoderm induction and L-R axis specification. The induction of *Xatv* by Xnr signaling in animal caps is consistent with the temporal appearance of *Xatv* after *Xnr* in normal embryogenesis. Our results, in addition to those from other laboratories, imply that mesendoderm induction *in vivo* is the result of a spatiotemporally coordinated balance between cell-autonomously activated inducers (e.g. Zorn et al., 1999), positive feedback Xnr relays (Osada and Wright, 1999), and negative feedback via Xnr-induced factors such as Xatv. Another general conclusion is that, in addition to Nodal signaling playing an essential role in mesendoderm induction in *Xenopus*, zebrafish and mice (Collignon et al., 1996; Osada and Wright, 1999; Piccolo et al., 1999; Zhou et al., 1993), the lefty/antivin factors seem to be conserved negative regulatory influences on this pathway (Bisgrove et al., 1999; Meno et al., 1999; Thisse and Thisse, 1999).

Asymmetric *Xnr1* expression during tailbud tadpole stages induces asymmetric *Xatv* expression, and a negative feedback loop through *Xatv*-mediated antagonism is again indicated by our observation that *Xatv* overexpression suppresses *Xnr1*/*XPitx2* expression in the left LPM. Overall, we suggest that induction of *Xatv* is important to limit the amount of mesoderm induction at gastrulation, and ensure the transient nature of 'left signals' within tailbud stage LPM that are essential for asymmetric morphogenesis.

Xatv – a lefty ortholog

The structure, function and expression pattern of *Xatv* argue that it is a true *lefty* ortholog. The level of identity between Xatv and mouse lefty1/lefty2 (approx. 35% over the whole protein) is lower than for other TGFβ-like factors such as BMP4, but similar to that between mammalian and frog nodal orthologs (approx. 36% overall). Two zebrafish *lefty*-like

genes, *zlft1* (which probably corresponds to *antivin*) and *zlft2*, were recently described (Bisgrove et al., 1999). From considerations of sequence and expression patterns, it is unclear whether mouse *lefty1* and *lefty2* are homologous to zebrafish *atv/zlft1* and *zlft2*, respectively, or if both pairs arose by species-specific gene duplication. It is similarly difficult to make definitive homology arguments for *Xatv*; while the *Xenopus* genome may contain additional *Xatv*/*lefty*-related genes, we only isolated the A/B pseudotetraploid copies of the same gene.

A general conclusion from our studies is that the *Xatv* expression pattern seems to comprise aspects of those of *lefty1*/*lefty2* and *zlft1*/*zlft2*, with additional species-specific domains. Like *lefty2* (Meno et al., 1997), *Xatv* is expressed during gastrulation in the area of mesoderm formation. *Xatv* becomes progressively restricted to the embryonic midline during neurulation and, following neural tube formation, axial *Xatv* expression marks the neural tube floorplate, notochord anlage, hypochord and dorsal endoderm. The symmetric expression of *Xatv* in the floorplate differs from the left floorplate expression of *lefty1* in mouse (Meno et al., 1997), but species-specific anatomical differences in this structure may contribute to this disparity. A requirement for full axial midline specification in the development of normal visceral asymmetry (Yost, 1998) may be connected to the role of lefty ligands in preventing 'left signals' crossing to the embryo's right side (Meno et al., 1998). *Xatv* expression within the axial midline may serve a similar function in *Xenopus*.

Two *Xatv* expression domains not noted for *lefty*/*atv* genes in fish or mouse are the left dorsal endoderm in the anterior of tailbud *Xenopus* embryos, and the hypochord. Both tissues have been linked to normal cardiovascular development in vertebrates. For example, extirpation of anterior endoderm in frogs decreases the frequency of beating heart tissue (Nascone and Mercola, 1995), and in mouse, it has been shown that GATA4-expressing endoderm is required for normal heart morphogenesis (Narita et al., 1997). An inductive role for the hypochord as a VEGF source has been proposed in dorsal aorta formation (Cleaver and Krieg, 1998). *Xatv* may modulate the function of signaling molecules involved in these activities, or additional inductive influences. In particular, we speculate that the left-sided dorsal endoderm expression in anterior regions may be connected to the heart and/or gut looping process.

Later in development, *Xatv* is asymmetrically expressed in the left LPM, like *atv/zlft1*, *zlft2*, and mouse *lefty2* (Bisgrove et al., 1999; Meno et al., 1997; Thisse and Thisse, 1999). Zebrafish *atv/zlft1* is also expressed very transiently in the left dorsal diencephalon; further examination will be necessary to determine if corresponding expression occurs in *Xenopus*.

Xatv and mesendodermal patterning

The ability of Xatv to suppress mesoderm induction by activin or Xnr2 in animal caps mimics the activities of zebrafish atv and mouse lefty in zebrafish embryos (Thisse and Thisse, 1999). *Xatv* is induced by Xnr signaling, and Xatv blocks induction by Xnrs. In the left LPM, Xatv can suppress *Xnr1* expression. These results are consistent with concurrent studies carried out in mouse and zebrafish (Meno et al., 1999). A parsimonious model to explain these observations is that negative feedback modulation by Xatv of the level of *Xnr* expression acts to regulate the amount of mesendoderm induced by Nodal/Xnr signaling, and/or affect its dorsoventral/anteroposterior character. This hypothesis is concordant with the expanded *nodal* expression and overproduction of nascent mesoderm in *lefty2* homozygous null mouse embryos. A good example of the modification of mesodermal character by Xatv is the reduction of organizer markers and appearance of *globin* expression induced by Xnr/Xatv coexpression (Fig. 4). Presumably, the precise regulation of the expression levels of these dose-dependent inducers and feedback antagonists is tightly linked to cell determination during gastrulation. It would thus be useful to understand the spatiotemporal distributions of these factors at the protein level in relation to high resolution embryonic fate maps.

Our observation that Xatv cannot neuralize ectoderm implies that it selectively antagonizes inducers like activin/Xnr, and not BMPs. In fact, Xatv might upregulate BMP activity in vivo, without necessarily affecting BMP transcription, if it were to reduce the Xnr-mediated induction of BMP inhibitors such as *chordin* and *noggin* (e.g. Osada and Wright, 1999; this paper). Future experiments will address whether Xatv antagonizes other mesendoderm inducers (e.g. derrière; Sun et al., 1999). Regarding *Xatv* expression in the anterior mesendoderm of neurula stage embryos (e.g. Fig. 2E), *Xnr4* is also expressed in this tissue (Joseph and Melton, 1997), suggesting a potential functional interaction between these two factors in anterior tissue specification.

As already suggested for zebrafish *atv/zlft1* (Bisgrove et al., 1999; Thisse and Thisse, 1999), Xatv might antagonize Xnr2 or activin in several ways: direct binding to the inducing ligand, stimulating an Xatv receptor followed by intracellular suppression, or by binding Xnr/activin receptors thereby preventing ligand access. Previous data are consistent with the latter mechanism (Meno et al., 1999; Thisse and Thisse, 1999). Our experiments suggest that there are differences in the ability of Xatv to suppress activin and Xnr2. For example, while 100 pg of *Xatv* RNA could completely suppress activin-mediated mesendodermal induction, the same dose reproducibly suppressed only the induction of organizer-specific markers by Xnr2 (Fig. 4). The relevance of this observation is currently unclear. Based upon work in fish and mouse, however, signaling by nodal (unlike activin) seems to require the presence of facilitating factors in the EGF-CFC family (Gritsman et al., 1999; Shen et al., 1997). Thus, the interactions of Xnr/Xatv with the receptor signaling system may be significantly different from those for activin/Xatv.

Absence of 'left' signals disrupts asymmetry

Right-sided *Xnr1* misexpression induces a mirror-image duplication of the left expression pattern of *Xatv* (Fig. 5; Table 1). This effect reiterates the inductive relationship between *Xnr* and *Xatv* in animal caps, and suggests a level of commonality between the processes of mesoderm induction and L-R specification. Conversely, left-sided *Xatv* overexpression downregulates or abrogates the endogenous asymmetric expression of *Xnr1* and *XPitx2* (Fig. 6; Tables 2, 3).

Our data indicating that Xatv acts to suppress 'left' pathways in *Xenopus* are largely concordant with functional studies of mouse lefty in chick embryos (Yoshioka et al., 1998). Implantation of cells producing lefty1 or lefty2 on either side of the node in stage 5 chick embryos led to the suppression of

left-sided *nodal* and *Pitx2* expression in approx. 50% of the embryos. In our case, in addition to the highly efficient abrogation of endogenous *Xnr1*/*XPitx2* expression caused by left-sided *Xatv* misexpression, a lower percentage (approx. 20%) of frog embryos showed bilateral absence of *Xnr1*/*XPitx2* expression in response to right-sided *Xatv* delivery. This variability between chick and frog may reflect the different misexpression methods used. In both species, the ability of right-sided delivery to suppress 'left' gene programs contralaterally may represent secretion across the midline, or an effect on tissues other than the LPM. The observation that placing lefty-expressing cells in the right LPM of older chick embryos (stage 7+) induced right-sided *Pitx2* expression (Yoshioka et al., 1998) is difficult to understand, since we did not detect this inductive property for Xatv in frog embryos. It is possible that this observation in chick represents a species-specific outcome, an indirect effect on the axial midline/node, or a non-physiological response related to the dose and/or timing of lefty misexpression.

L-R morphogenesis and left signals

The current explanation for the way that bilateral LPM expression of *nodal/Xnr1* and *Pitx2*/*XPitx2* leads to situs randomization involves a hypothetical 'stochastic selection step', at the global or organ-specific level, in which either the endogenous or the ectopic left signal becomes dominant and triggers normal or reversed asymmetric morphogenesis (e.g. Sampath et al., 1997).

The absence of 'left' *Xnr1/XPitx2* signals seen in *Xatv*-injected *Xenopus* embryos may lead to organ dysmorphogenesis via an intermediate bilateral 'right' state (right isomerism), and addressing this point will require further study, including the development of additional molecular markers and higher resolution morphological criteria for organ situs in *Xenopus*. In mouse, specific anatomical criteria (some of which, e.g. lung lobation, are not found in frogs) allow fairly detailed characterization of non-global situs alterations, for example, pulmonary or thoracic isomerisms, and more complex cardiovascular system alterations such as those seen in *lefty1* mutants (Meno et al., 1998). In contrast, here we have only broadly categorized the cardiac and visceral orientation in *Xenopus* as normal, reversed, or indeterminate. Despite this caveat, the ability of left-sided *Xatv* overexpression to suppress *Xnr1*/*XPitx2* expression in the left LPM, and the inability of these embryos to undergo normal L-R asymmetric morphogenesis, suggests that *Xatv* functions *in vivo* to downregulate the expression and activity of 'left' signals (*Xnr1, XPitx2*) that are essential for L-R axis development.

One important possibility that is hard to rule out is that the dysmorphogenesis seen in a low percentage of embryos receiving Xatv on the right side reflects interference with a right side gene cascade (see Introduction), rather than contralateral secretion and interference with 'left pathways'. While the lack of suitable markers is again a hindrance, the failure of right-sided Xatv expression to induce *Xnr1* or *XPitx2* on the right, or to generate a high incidence of bilaterally absent Xnr1/XPitx2 expression, does suggest that such right side interference cannot produce left isomerism.

Thus, our data overall fit better with the hypothesis that 'left' signals (involving *Xnr1*/*XPitx2*), which can be blocked by Xatv misexpression, are essential for asymmetric morphogenesis. The right pulmonary isomerism observed in *Pitx2*$^{-/-}$ mice (Lin et al., 1999) supports this idea. On the other hand, treatment of mouse embryos with retinoic acid antagonists, which apparently blocks *lefty*, *nodal* and *Pitx2* expression, causes situs randomization (Chazaud et al., 1999). An important experiment will be to test the effect on L-R morphogenesis of selectively removing left-sided *nodal* expression from mouse embryos, perhaps by conditional gene inactivation.

Although we cannot determine when the Xatv-mediated block to the left gene cascade occurs, when assayed at stages corresponding to the earliest phase of asymmetric *Xnr1* expression, left-sided *Xatv* delivery causes bilateral absence of *Xnr1* at an approx. 70% frequency (similar to the incidence in Table 3; data not shown). It will therefore be important to determine how Xnr1-Xatv function is linked to earlier steps in L-R axis specification, including the actions of the putative L-R coordinator, Vg1 (Hyatt and Yost, 1998). Additional outstanding issues include whether Xatv affects only the LPM, and/or organizer-proximate L-R specification processes occurring around gastrulation, and if it affects *Xnr1* alone and/or additional signals.

Xatv/lefty function in asymmetric morphogenesis

We present a model for Xatv function in L-R axis determination in frog embryos (Fig. 8). The expression domain of *Xnr1* in the left LPM changes rapidly with time. It is first activated posteriorly, then rapidly expands anteriorly through the left LPM and begins to undergo retraction posteriorly. In anterior regions, expression shifts forward to abut the heart anlage (Lowe et al., 1996). The mechanisms regulating this dynamic movement, which runs against the rostral-caudal direction of tissue specification, are unknown, but the degree of shifting is too large to be accounted for by cell migration. Primarily, there must be spreading of *Xnr1* expression anteriorly and progressive downregulation posteriorly. In our model, the inducer/inhibitor relationship between *Xnr1*/*Xatv* constitutes a self-regulating negative feedback loop (Fig. 8). First, *Xnr1* induces *Xatv* and *XPitx2* expression in a spatiotemporal pattern following that of *Xnr1*. With a built-in delay, the induction of Xatv then inactivates *Xnr1* expression in the same spatiotemporal manner, beginning posteriorly. This feedback loop would serve to prevent the inappropriate spread of Xnr signals through autoactivation (Osada and Wright, 1999; Saijoh et al., 2000; S. Osada and C. V. E. W., unpublished data), and ensure transient *Xnr1* expression as a trigger of asymmetric morphogenesis. Since *XPitx2* expression is prolonged compared to *Xnr1*, it presumably escapes Xatv-mediated inactivation. As a transcription factor, XPitx2 then begins to stabilize the initially labile L-R asymmetries by activating, together with other factors, the downstream effector programs that directly drive asymmetric morphogenesis within the organ anlagen. At later stages, the *Xatv* expression within the developing heart, after the termination of asymmetric *Xnr1* transcription, suggests that Xatv modulates inductive influences of perdurant Xnrs, or that *Xatv* expression is also induced or maintained by other signaling molecules involved in later aspects of L-R differential morphogenesis.

Aspects of this model may be conserved in other vertebrates. In chick and mouse, examination of the published literature indicates that *nodal* expression also spreads in a stereotypical fashion through the LPM (Collignon et al., 1996; Logan et al.,

1998; Lowe et al., 1996; Rodriguez Esteban et al., 1999). While the anterior shift seems similar to that observed for *Xnr1*, substantial spreading occurs posteriorly from the point where *nodal* LPM expression is initiated nearest the node. This apparent inconsistency may be related to differences in the coordination of body axis extension and L-R asymmetric morphogenesis. Experiments to test our model will include rigorous comparisons of the expression domains of *lefty*/*antivin* genes with respect to *nodal* by double label in situ hybridization experiments.

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REFERENCES

Bisgrove, B. W., Essner, J. J. and Yost, H. J. (1999). Regulation of midline development by antagonism of lefty and nodal signaling. *Development* **126**, 3253-3262.

Boettger, T., Wittler, L. and Kessel, M. (1999). FGF8 functions in the specification of the right body side of the chick. *Curr. Biol.* **9**, 277-280.

Bouwmeester, T., Kim, S., Sasai, Y., Lu, B. and De Robertis, E. M. (1996). Cerberus is a head-inducing secreted factor expressed in the anterior endoderm of Spemann's organizer. *Nature* **382**, 595-601.

Campione, M., Steinbeisser, H., Schweickert, A., Deissler, K., van Bebber, F., Lowe, L. A., Nowotschin, S., Viebahn, C., Haffter, P., Kuehn, M. R. et al. (1999). The homeobox gene *Pitx2*: mediator of asymmetric left-right signaling in vertebrate heart and gut looping. *Development* **126**, 1225-1234.

Chang, C., Wilson, P. A., Mathews, L. S. and Hemmati-Brivanlou, A. (1997). A *Xenopus* type I activin receptor mediates mesodermal but not neural specification during embryogenesis. *Development* **124**, 827-837.

Chazaud, C., Chambon, P. and Dolle, P. (1999). Retinoic acid is required in the mouse embryo for left-right asymmetry determination and heart morphogenesis. *Development* **126**, 2589-2596.

Cleaver, O. and Krieg, P. A. (1998). VEGF mediates angioblast migration during development of the dorsal aorta in *Xenopus. Development* **125**, 3905-3914.

Collignon, J., Varlet, I. and Robertson, E. J. (1996). Relationship between asymmetric nodal expression and the direction of embryonic turning. *Nature* **381**, 155-158.

Condie, B. G., Brivanlou, A. H. and Harland, R. M. (1990). Most of the homeobox-containing Xhox 36 transcripts in early Xenopus embryos cannot encode a homeodomain protein. *Mol. Cell Biol.* **10**, 3376-3385.

Drysdale, T. A., Tonissen, K. F., Patterson, K. D., Crawford, M. J. and Krieg, P. A. (1994). Cardiac troponin I is a heart-specific marker in the *Xenopus* embryo: expression during abnormal heart morphogenesis. *Dev. Biol.* **165**, 432-441.

Graff, J. M., Thies, R. S., Song, J. J., Celeste, A. J. and Melton, D. A. (1994). Studies with a Xenopus BMP receptor suggest that ventral mesoderm- inducing signals override dorsal signals in vivo. *Cell* **79**, 169-179.

Gritsman, K., Zhang, J., Cheng, S., Heckscher, E., Talbot, W. S. and Schier, A. F. (1999). The EGF-CFC protein one-eyed pinhead is essential for nodal signaling. *Cell* **97**, 121-132.

Harland, R. M. (1991). In situ hybridization: an improved whole-mount method for Xenopus embryos. *Methods Cell Biol.* **36**, 685-695.

Harvey, R. P. (1998). Links in the left/right axial pathway. *Cell* **94**, 273-276.

Hyatt, B. A. and Yost, H. J. (1998). The left-right coordinator: the role of Vg1 in organizing left-right axis formation. *Cell* **93**, 37-46.

Johnson, A. D. and Krieg, P. A. (1994). pXeX, a vector for efficient expression of cloned sequences in Xenopus embryos. *Gene* **147**, 223-226.

Jones, C. M., Kuehn, M. R., Hogan, B. L., Smith, J. C. and Wright, C. V. (1995). Nodal-related signals induce axial mesoderm and dorsalize mesoderm during gastrulation. *Development* **121**, 3651-3662.

Joseph, E. M. and Melton, D. A. (1997). Xnr4: a Xenopus nodal-related gene expressed in the Spemann organizer. *Dev. Biol.* **184**, 367-372.

Kay, B. K. and Peng, H. B. (1991). Xenopus laevis: Practical Uses in Cell and Molecular Biology. In *Methods in Cell Biology 36*. Academic Press, Inc.

Kingsley, D. M. (1994). The TGF-beta superfamily: new members, new receptors, and new genetic tests of function in different organisms. *Genes Dev.* **8**, 133-146.

Kosaki, K. and Casey, B. (1998). Genetics of human left-right axis malformations. *Semin. Cell Dev. Biol.* **9**, 89-99.

Krieg, P. A., Varnum, S. M., Wormington, W. M. and Melton, D. A. (1989). The mRNA encoding elongation factor 1-alpha (EF-1 alpha) is a major transcript at the midblastula transition in Xenopus. *Dev. Biol.* **133**, 93-100.

Lemaire, P. and Gurdon, J. B. (1994). A role for cytoplasmic determinants in mesoderm patterning: cell- autonomous activation of the goosecoid and Xwnt-8 genes along the dorsoventral axis of early *Xenopus* embryos. *Development* **120**, 1191-1199.

Levin, M., Johnson, R. L., Stern, C. D., Kuehn, M. and Tabin, C. (1995). A molecular pathway determining left-right asymmetry in chick embryogenesis. *Cell* **82**, 803-814.

Lin, C. R., Kioussi, C., O'Connell, S., Briata, P., Szeto, D., Liu, F., Izpisua-Belmonte, J. C. and Rosenfeld, M. G. (1999). Pitx2 regulates lung asymmetry, cardiac positioning and pituitary and tooth morphogenesis. *Nature* **401**, 279-282.

Logan, M., Pagan-Westphal, S. M., Smith, D. M., Paganessi, L. and Tabin, C. J. (1998). The transcription factor Pitx2 mediates situs-specific morphogenesis in response to left-right asymmetric signals. *Cell* **94**, 307-317.

Lowe, L. A., Supp, D. M., Sampath, K., Yokoyama, T., Wright, C. V., Potter, S. S., Overbeek, P. and Kuehn, M. R. (1996). Conserved left-right asymmetry of nodal expression and alterations in murine situs inversus. *Nature* **381**, 158-161.

Meno, C., Gritsman, K., Ohishi, S., Ohfuji, Y., Heckscher, E., Mochida, K., Shimono, A., Kondoh, H., Talbot, W. S., Robertson, E. J. et al. (1999). Mouse Lefty2 and zebrafish antivin are feedback inhibitors of nodal signaling during vertebrate gastrulation. *Mol. Cell* **4**, 287-298.

Meno, C., Ito, Y., Saijoh, Y., Matsuda, Y., Tashiro, K., Kuhara, S. and Hamada, H. (1997). Two closely-related left-right asymmetrically expressed genes, lefty-1 and lefty-2: their distinct expression domains, chromosomal linkage and direct neuralizing activity in Xenopus embryos. *Genes Cells* **2**, 513-524.

Meno, C., Saijoh, Y., Fujii, H., Ikeda, M., Yokoyama, T., Yokoyama, M., Toyoda, Y. and Hamada, H. (1996). Left-right asymmetric expression of the TGF beta-family member lefty in mouse embryos. *Nature* **381**, 151-155.

Meno, C., Shimono, A., Saijoh, Y., Yashiro, K., Mochida, K., Ohishi, S., Noji, S., Kondoh, H. and Hamada, H. (1998). lefty-1 is required for left-right determination as a regulator of lefty-2 and nodal. *Cell* **94**, 287-297.

Meyers, E. N. and Martin, G. R. (1999). Differences in left-right axis pathways in mouse and chick: functions of FGF8 and SHH. *Science* **285**, 403-406.

Mochizuki, T., Saijoh, Y., Tsuchiya, K., Shirayoshi, Y., Takai, S., Taya, C., Yonekawa, H., Yamada, K., Nihei, H., Nakatsuji, N. et al. (1998). Cloning of inv, a gene that controls left/right asymmetry and kidney development. *Nature* **395**, 177-181.

Narita, N., Bielinska, M. and Wilson, D. B. (1997). Cardiomyocyte differentiation by GATA-4-deficient embryonic stem cells. *Development* **124**, 3755-3764.

Nascone, N. and Mercola, M. (1995). An inductive role for the endoderm in *Xenopus* cardiogenesis. *Development* **121**, 515-523.

Nieuwkoop, P. D. and Faber, J. (1967). Normal table of *Xenopus laevis* (Daudin). Amsterdam: North Holland Publishing Company.

Osada, S. I. and Wright, C. V. (1999). *Xenopus* nodal-related signaling is essential for mesendodermal patterning during early embryogenesis. *Development* **126**, 3229-3240.

Patel, K., Isaac, A. and Cooke, J. (1999). Nodal signalling and the roles of the transcription factors SnR and pitx2 in vertebrate left-right asymmetry. *Curr. Biol.* **9**, 609-612.

Piccolo, S., Agius, E., Leyns, L., Bhattacharyya, S., Grunz, H., Bouwmeester, T. and De Robertis, E. M. (1999). The head inducer Cerberus is a multifunctional antagonist of Nodal, BMP and Wnt signals. *Nature* **397**, 707-710.

Piedra, M. E., Icardo, J. M., Albajar, M., Rodriguez-Rey, J. C. and Ros, M. A. (1998). Pitx2 participates in the late phase of the pathway controlling left- right asymmetry. *Cell* **94**, 319-324.

Rodriguez Esteban, C., Capdevila, J., Economides, A. N., Pascual, J., Ortiz, A. and Izpisua Belmonte, J. C. (1999). The novel Cer-like protein Caronte mediates the establishment of embryonic left-right asymmetry. *Nature* **401**, 243-251.

Ryan, A. K., Blumberg, B., Rodriguez-Esteban, C., Yonei-Tamura, S., Tamura, K., Tsukui, T., de la Pena, J., Sabbagh, W., Greenwald, J., Choe, S. et al. (1998). Pitx2 determines left-right asymmetry of internal organs in vertebrates. *Nature* **394**, 545-551.

Saijoh, Y., Adachi, H., Sakuma, R., Yeol-yeo, C., Watanabe, M., Hashiguchi, H., Mochida, K., Ohishi, S., Kawabata, M., Miyazono, K. et al. (2000). Left-right asymmetric expression of lefty2 and nodal is induced by a signaling pathway that includes the transcription factor FAST2. *Mol. Cell.* **5**, 35-47.

Sampath, K., Cheng, A. M., Frisch, A. and Wright, C. V. (1997). Functional differences among *Xenopus* nodal-related genes in left-right axis determination. *Development* **124**, 3293-3302.

Sasai, Y., Lu, B., Piccolo, S. and De Robertis, E. M. (1996). Endoderm induction by the organizer-secreted factors chordin and noggin in Xenopus animal caps. *EMBO J.* **15**, 4547-4555.

Sasai, Y., Lu, B., Steinbeisser, H. and De Robertis, E. M. (1995). Regulation of neural induction by the Chd and Bmp-4 antagonistic patterning signals in *Xenopus*. *Nature* **376**, 333-336.

Schneider, A., Mijalski, T., Schlange, T., Dai, W., Overbeek, P., Arnold, H. H. and Brand, T. (1999). The homeobox gene it NKX3.2 is a target of left-right signalling and is expressed on opposite sides in chick and mouse embryos. *Curr. Biol.* **9**, 911-914.

Shen, M. M., Wang, H. and Leder, P. (1997). A differential display strategy identifies Cryptic, a novel EGF-related gene expressed in the axial and lateral mesoderm during mouse gastrulation. *Development* **124**, 429-442.

Smith, W. C., McKendry, R., Ribisi, S., Jr. and Harland, R. M. (1995). A nodal-related gene defines a physical and functional domain within the Spemann organizer. *Cell* **82**, 37-46.

Sun, B. I., Bush, S. M., Collins-Racie, L. A., LaVallie, E. R., DiBlasio-Smith, E. A., Wolfman, N. M., McCoy, J. M. and Sive, H. L. (1999). derriere: a TGF-β family member required for posterior development in *Xenopus*. *Development* **126**, 1467-1482.

Supp, D. M., Witte, D. P., Potter, S. S. and Brueckner, M. (1997). Mutation of an axonemal dynein affects left-right asymmetry in inversus viscerum mice. *Nature* **389**, 963-966.

Thisse, C. and Thisse, B. (1999). Antivin, a novel and divergent member of the TGFβ superfamily, negatively regulates mesoderm induction. *Development* **126**, 229-240.

Wilson, P. A. and Melton, D. A. (1994). Mesodermal patterning by an inducer gradient depends on secondary cell- cell communication. *Curr. Biol.* **4**, 676-686.

Yokouchi, Y., Vogan, K. J., Pearse, R. V., 2nd and Tabin, C. J. (1999). Antagonistic signaling by Caronte, a novel Cerberus-related gene, establishes left-right asymmetric gene expression. *Cell* **98**, 573-583.

Yoshioka, H., Meno, C., Koshiba, K., Sugihara, M., Itoh, H., Ishimaru, Y., Inoue, T., Ohuchi, H., Semina, E. V., Murray, J. C. et al. (1998). Pitx2, a bicoid-type homeobox gene, is involved in a lefty-signaling pathway in determination of left-right asymmetry. *Cell* **94**, 299-305.

Yost, H. J. (1998). The genetics of midline and cardiac laterality defects. *Curr. Opin. Cardiol.* **13**, 185-189.

Zhou, X., Sasaki, H., Lowe, L., Hogan, B. L. and Kuehn, M. R. (1993). Nodal is a novel TGF-beta-like gene expressed in the mouse node during gastrulation. *Nature* **361**, 543-547.

Zhu, L., Marvin, M. J., Gardiner, A., Lassar, A. B., Mercola, M., Stern, C. D. and Levin, M. (1999). Cerberus regulates left-right asymmetry of the embryonic head and heart. *Curr. Biol.* **9**, 931-938.

Zorn, A. M., Butler, K. and Gurdon, J. B. (1999). Anterior endomesoderm specification in Xenopus by Wnt/beta-catenin and TGF-beta signalling pathways. *Dev. Biol.* **209**, 282-297.