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Distinct *Xenopus* Nodal ligands sequentially induce mesendoderm and control gastrulation movements in parallel to the Wnt/PCP pathway

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SUMMARY

The vertebrate body plan is established in two major steps. First, mesendoderm induction singles out prospective endoderm, mesoderm and ectoderm progenitors. Second, these progenitors are spatially rearranged during gastrulation through numerous and complex movements to give rise to an embryo comprising three concentric germ layers, polarised along dorsoventral, anteroposterior and left-right axes. Although much is known about the molecular mechanisms of mesendoderm induction, signals controlling gastrulation movements are only starting to be revealed. In vertebrates, Nodal signalling is required to induce the mesendoderm, which has precluded an analysis of its potential role during the later process of gastrulation. Using time-dependent inhibition, we show that in *Xenopus*, Nodal signalling plays sequential roles in mesendoderm induction and gastrulation movements. Nodal activity is necessary for convergent extension in axial mesoderm and for head mesoderm migration. Using morpholino-mediated knockdown, we found that the Nodal ligands Xnr5 and Xnr6 are together required for mesendoderm induction, whereas Xnr1 and Xnr2 act later to control gastrulation movements. This control is operated via the direct regulation of key movement-effector genes, such as *papc*, *has2* and *pdgfrα*. Interestingly, however, Nodal does not appear to mobilise the Wnt/PCP pathway, which is known to control cell and tissue polarity. This study opens the way to the analysis of the genetic programme and cell behaviours that are controlled by Nodal signalling during vertebrate gastrulation. It also provides a good example of the sub-functionalisation that results from the expansion of gene families in evolution.

KEY WORDS: *Xenopus*, Nodal, Mesendoderm induction, Gastrulation, Convergent extension, Migration, Intercalation, Wnt/PCP

INTRODUCTION

Gastrulation entails a set of morphogenetic processes that generate the external form of developing animals as well as the internal organisation of organ primordia. During the course of gastrulation, the precursors of the three germ layers, the endoderm, mesoderm and ectoderm, are repositioned from the surface of the blastula, such that at the end of this process the mesoderm is placed between the internal endoderm and the superficial ectoderm (Leptin, 2005; Solnica-Krezel, 2005). The germ-layer precursors are specified prior to gastrulation through a process called mesendoderm induction. In all vertebrate model systems, mesendoderm induction is a zygotic event that mobilises several signalling pathways, among which the Nodal family plays the most important part. Inhibition of Nodal activity by genetic mutation or expression of antimorphic constructs prevents mesendoderm specification in frog, fish and mouse (Kimelman, 2006; Shen, 2007). Consequently, Nodal-deficient embryos do not gastrulate, as they lack the tissues that normally undergo internalisation. Thus, it is generally assumed that mesendoderm induction and gastrulation are jointly controlled by Nodal signalling, although a demonstration of a direct role in the second process is still lacking.

Nodal ligands, which belong to the TGFβ superfamily, signal through a complex of type I serine-threonine kinase receptors (Alk4 and Alk7) and type II receptors (Shen, 2007). In contrast to other TGFβ members, such as Activin, the reception of Nodal also depends on the presence of a co-receptor of the EGF-CFC family (Shen and Schier, 2000). The receptor complex, when bound to Nodal ligands, phosphorylates Smad2 and Smad3, which in turn bind to Smad4, enter the nucleus and control gene expression in combination with additional transcription factors, such as FoxH1 and Mixer (Shen, 2007). The pathway is regulated at multiple points, in particular by extracellular antagonists, such as Lefty proteins, which bind to Nodal ligands and the EGF-CFC co-receptor, thereby preventing the formation of receptor complexes (Shen, 2007).

The Nodal gene family pre-dates the separation between protostomians and deuterostomians (Grande and Patel, 2009) and has been subject to considerable diversification during the course of evolution. Whereas there is only one Nodal family member in chick and mouse, there are three in zebrafish and six in *Xenopus* (Xnr1-6, for *Xenopus* Nodal-related). In *Xenopus*, which serves as a model in our study, five ligands can induce mesendoderm in naïve ectoderm; Xnr3 has lost this property owing to the mutation of a conserved cysteine residue (Hansen et al., 1997; Jones et al., 1995; Joseph and Melton, 1997; Takahashi et al., 2000). *Xnr4* shows restricted expression in notochord precursors and is unlikely to play a major role in general mesendoderm induction, although it may participate in patterning (Joseph and Melton, 1997). *Xnr1*, *Xnr2*, *Xnr5* and *Xnr6* are all expressed in deep vegetal cells of the blastula, where the endogenous mesendoderm inducers are produced, although expression of the latter two ligands starts notably earlier (Jones et al., 1995; Takahashi et al., 2000; Yang et al., 2002). During early gastrulation, *Xnr1* and *Xnr2* remain expressed in circumblastoporal tissues, whereas expression of *Xnr5* and *Xnr6* decreases dramatically (Jones et al., 1995; Takahashi et al., 2006; Takahashi et al., 2000). Direct functional evidence linking specific Xnrs to mesendoderm induction or to gastrulation is still missing, as

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previous studies involved the use of cleavage mutants that act in a dominant-negative manner on several members of the family (Onuma et al., 2002; Osada and Wright, 1999).

Gastrulation consists of a suite of movements that each contribute to specific changes in tissue and embryo shape. Among these movements, convergence and extension are certainly the most studied and the best understood in molecular and cell biological terms (Keller and Shook, 2004; Solnica-Krezel, 2005). Convergence narrows embryonic tissues mediolaterally, whereas extension movements elongate them anteroposteriorly. In the frog chordamesoderm and in paraxial mesoderm these two movements are coupled in a process termed convergent extension. The driving force for convergent extension is intercalation behaviour, whereby cells exhibit polarised protrusive activity that allows them to exert traction and to wedge between each other, while maintaining the stiffness of the remodelling tissue. Convergent extension depends extensively on the integrity of the non-canonical Wnt pathway, which exhibits analogies with the fly planar cell polarity (PCP) pathway (Solnica-Krezel, 2005; Wallingford et al., 2002). In both frog and fish, the non-canonical Wnt pathway is triggered by Wnt11, which signals through Frizzled receptors to recruit Dishevelled (Dsh) at the cell membrane and activate downstream effectors, including small GTPases of the Rho family, Rho kinase and JNK (Solnica-Krezel, 2005).

Directed cell migration is another type of cell behaviour that plays an important role during gastrulation. It is best exemplified in the prechordal mesoderm that migrates as a loosely coherent mass of cells onto the roof of the blastocoele and towards the animal pole (Keller and Shook, 2004; Solnica-Krezel, 2005). This directed migratory behaviour involves the polarised protrusive activity of mesendodermal cells, as well as the production by ectodermal cells of fibronectin-rich extracellular matrix and of chemoattractant molecules, such as PDGF (Nagel et al., 2004; Winklbauer and Keller, 1996).

We show here that Xnr1 and Xnr2 are required for convergent extension in axial and paraxial mesoderm, as well as for migration of rostral mesoderm. We propose that these morphogenetic functions involve the production of a transcriptional movement programme that follows the early mesendoderm programme, which is controlled by Xnr5 and Xnr6.

MATERIALS AND METHODS

Embryos, explants and injections

Xenopus eggs obtained from NASCO females were fertilised in vitro, dejellied, cultured and injected as described (Carnac et al., 1996). Synthetic capped RNAs were produced with the Ambion mMessage mMachine Kit. References for our expression constructs are: *Xnr1* and *Xnr2* (Jones et al., 1995); *Xnr5* and *Xnr6* alleles (Takahashi et al., 2006; Takahashi et al., 2000); *dsh-GFP* (Wallingford et al., 2000); and *mcherry* (Nie and Chang, 2007). Dorsal marginal zone (DMZ) explants were taken at early gastrula stage 10, and animal caps were explanted at blastula stage 8.5; both were cultured in 1× MBS supplemented with gentamycin. Prechordal mesoderm explants were prepared at stage 10.25 and cultured in 1× MBS; spreading on slides pre-coated overnight at 23°C with Fibronectin (Sigma, 10 μg/ml) was analysed after 30 minutes and 3 hours. Distance and area measurements on explants were made with Axiovision digital image software (Zeiss). For statistical analysis we applied a Kruskal-Wallis test under the open-source R project environment. Morpholino antisense oligonucleotides (MOs) were purchased from GeneTools, resuspended in sterile water at 10 μg/μl, and further diluted prior to injection. The Xnr1 MOa was reported previously (Toyoizumi et al., 2005). New MOs used in this study were:

Xnr1 MOb, 5′-GCACTGCTGATCTCTCTTTCAGGGA-3′;
Xnr2 MOa, 5′-CTAGGCTTGCCATCTCTGGAGAG-3′;
Xnr2 MOb, 5′-CTGTATTGATTGTCTGACTCTTCCA-3′;
Xnr5 MOa, 5′-AGATAAAGCCTAGCACAGCCATATC-3′;
Xnr5 MOb, 5′-CCATATCTGGAGAGGGAAGCTTCCA-3′;
Xnr6 MOa, 5′-CAAGACTAAGTTCACTAGGGCCATC-3′; and
Xnr6 MOb, 5′-TTCTCTTGGAGCTGGATCTTGTTCT-3′.

Fixable fluorescein lysine dextran (FLDx, 2.5 ng/cell) was co-injected with MOs to sort properly injected live embryos, and anti-fluorescein immunodetection was performed to trace the distribution of MOs in fixed embryos. All injections were performed at least twice to establish reproducibility.

Chemical and protein treatments

SB-505124 (Sigma) was dissolved in DMSO (to 20 mM) and diluted in 0.1× MBS for whole-embryo treatments or in 1× MBS for explant cultures. SU5402 was used as described (Delaune et al., 2005). Recombinant mouse Nodal and mouse Lefty proteins (R&D Systems) were resuspended as recommended by the provider, and injected into the blastocoele of embryos at blastula or gastrula stages at 5 or 10 ng/embryo. For animal caps, recombinant human Activin A (R&D Systems, 5 ng/ml) was used in 1× MBS. Cycloheximide (CHX) treatment (10 μg/ml) was started 45 minutes prior to the addition of Nodal or Activin to avoid any delay of action, and treatment was continued for 2.5 hours at 18°C.

Stainings

Whole-mount in situ hybridisation (WISH) with digoxygenin-labelled probes (Roche) was carried out as described (Marchal et al., 2009). Antisense riboprobes for *sox17α* (Hudson et al., 1997), *Xbra* (Smith et al., 1991), *goosecoid* (Yasuo and Lemaire, 2001), *Xnot-2* (Gont et al., 1993), *Xlim-1* (Taira et al., 1994), *sox2* (Mizuseki et al., 1998), *foxI1e* (Mir et al., 2007), *Xwnt11* (Tada and Smith, 2000), *rnd1* (Wunnenberg-Stapleton et al., 1999), *papc* (Kim et al., 1998), *pdgfrα* (Ho et al., 1994), *has2* (Nardini et al., 2004) and *camello* (Popsueva et al., 2001) were prepared as described in the respective references.

Immunohistochemistry using MZ15 and 12.101 monoclonal antibodies was performed as described (Carnac et al., 1996). We used a rabbit anti-GFP antibody (Torrey Pines Biolabs, 1/400) to reveal the Dsh-GFP fusion protein, and a secondary antibody coupled to Alexa 488 (Molecular Probes). Rhodamine phalloidin staining (Invitrogen, 5 units/ml in PBS containing 0.1% Triton X-100 and 1% BSA) was performed on sagittally hemisected embryos. Stained embryos were embedded in 4% agarose in PBS and 50 μm sections prepared with a vibration microtome (Microm HMV650V). Sections were mounted in Fluoromount (Fluoprobes) and imaged with a Zeiss Apotome Imager Z1 microscope.

Quantitative RT-PCR and western blotting

For quantitative RT-PCR (Q-PCR), primer sequences and annealing temperatures are presented in Table S1 in the supplementary material. Total RNAs were extracted with the RNeasy Mini Kit (Qiagen), cDNAs were synthesized using Superscript II reverse transcriptase (Invitrogen), and amplifications were performed in the presence of SYBR Green mix (Invitrogen) on a iQ5 machine (Bio-Rad). Annealing was for 15 seconds and elongation at 72°C for 45 seconds.

For western blotting, embryos were snap-frozen at stage 10 and processed as described (Batut et al., 2007). We used rabbit anti-pSmad2 (Cell Signaling, 1/1000), mouse anti-Smad2 (BD Biosciences, 1/1000) and mouse anti-α-tubulin (Sigma, 1/10,000) antibodies. For staining, we used HRP-coupled secondary antibodies and the ECL Plus chemiluminescent detection system (GE Healthcare). Exposed films were scanned and ImageJ software (NIH) was used to quantify signals. Experiments were repeated two or three times.

RESULTS

Nodal signalling is required for gastrulation independently of its earlier function in mesendoderm induction

In *Xenopus*, blocking Nodal signalling by early overexpression of antimorphic constructs prevents mesendoderm formation. We reasoned that late inhibition could perhaps allow us to test for a role of Nodal signalling in gastrulation without impairing Nodal function

in mesendoderm induction. In order to inhibit this pathway in a temporally controlled manner, we employed a pharmacological inhibitor of Alk4/5/7 receptors, SB-505124 (Hagos and Dougan, 2007), as well as a recombinant Lefty protein, which antagonises Nodal but not Activin ligands. We first determined the concentration of the drug that most efficiently repressed early mesendoderm marker gene expression. The endoderm marker *sox17α*, the mesendoderm marker *goosecoid* and the mesoderm marker *Xbra* were most severely repressed in embryos incubated from the 4-cell stage in 200 μM SB-505124 (see Fig. S1A,B in the supplementary material). By contrast, the ectoderm marker *foxI1e*, which is negatively regulated by Nodal signalling (Mir et al., 2007), was dramatically upregulated, confirming that transcription is maintained in the presence of the drug. In all subsequent experiments, we applied 200 μM SB-505124 on whole embryos and 50 μM on explants. When the drug was added at late blastula stage 9 (2 hours before the onset of gastrulation at stage 10), all mesendoderm markers tested were expressed in their normal territories and at normal levels in early gastrulae, as revealed by WISH and Q-PCR analyses (Fig. 1A,B; see Fig. S2A in the supplementary material). Likewise, when Lefty was injected prior to the mid-blastula transition (MBT), mesendoderm gene expression was severely downregulated, whereas injection at stage 9 had no effect (Fig. 1A,B; see Fig. S2A in the supplementary material). This apparent lack of mesendoderm gene repression could have been due to the overall shorter duration of inhibition. We thus applied SB-505124 for a period of 2 hours, starting either at MBT or at late blastula stage 9. *Xbra*, *goosecoid* and *sox17α* were repressed when inhibition started at stage 8, but not at stage 9 (see Fig. S2B in the supplementary material). We noted, however, that when inhibition was started at stage 8 it caused a less severe repression of these markers than when inhibition was started at the 4-cell stage, suggesting that Alk4/5/7 signalling might be active prior to MBT. We analysed the levels of phosphorylated Smad2 (pSmad2) at the onset of gastrulation as another readout of Alk4/5/7 signalling. SB-505124 applied at the 4-cell stage caused an over 80% reduction in pSmad2 levels, whereas treatment at stage 9 caused 40% reduction. A similar trend was observed in response to Lefty injection (Fig. 1C). It is known that the pool of pSmad2 remains elevated for several hours after activation by the ligand (Bourillot et al., 2002). Thus, pSmad2 activated up to stage 9 is likely to constitute the majority of the pool that is detectable at stage 10, which explains the weaker effect of stage 9 inhibition. Overall, the above results indicate that the period of requirement for Nodal activity in mesendoderm induction ends ~2 hours before the onset of gastrulation. We next asked whether Nodal inhibition past this period prevented gastrulation.

Embryos subjected to SB-505124 or to Lefty from stage 9 or 10 showed permanent defective blastopore closure, indicating that gastrulation did not occur normally (Fig. 2A; see Fig. S3A in the supplementary material). This effect was more severe when inhibition started at stage 9. By contrast, embryos gastrulated properly when Nodal inhibition was started at mid-gastrula stage 11. Staining for early markers revealed that prechordal mesoderm (*goosecoid*, *Xlim-1*) and chordamesoderm (*Xbra*, *Xnot-2*, *Xlim-1*) were present but abnormally positioned, as is usually seen when gastrulation is impaired independently of mesendoderm induction (Djiane et al., 2000; Ogata et al., 2007; Tada and Smith, 2000) (Fig. 2A,B; see Fig. S3A,B in the supplementary material). Further supporting this contention, notochord (MZ15) and muscle differentiation (12.101) were visible in tadpoles subjected to SB-505124 or Lefty protein at the late blastula stage (Fig. 2C; see Fig.

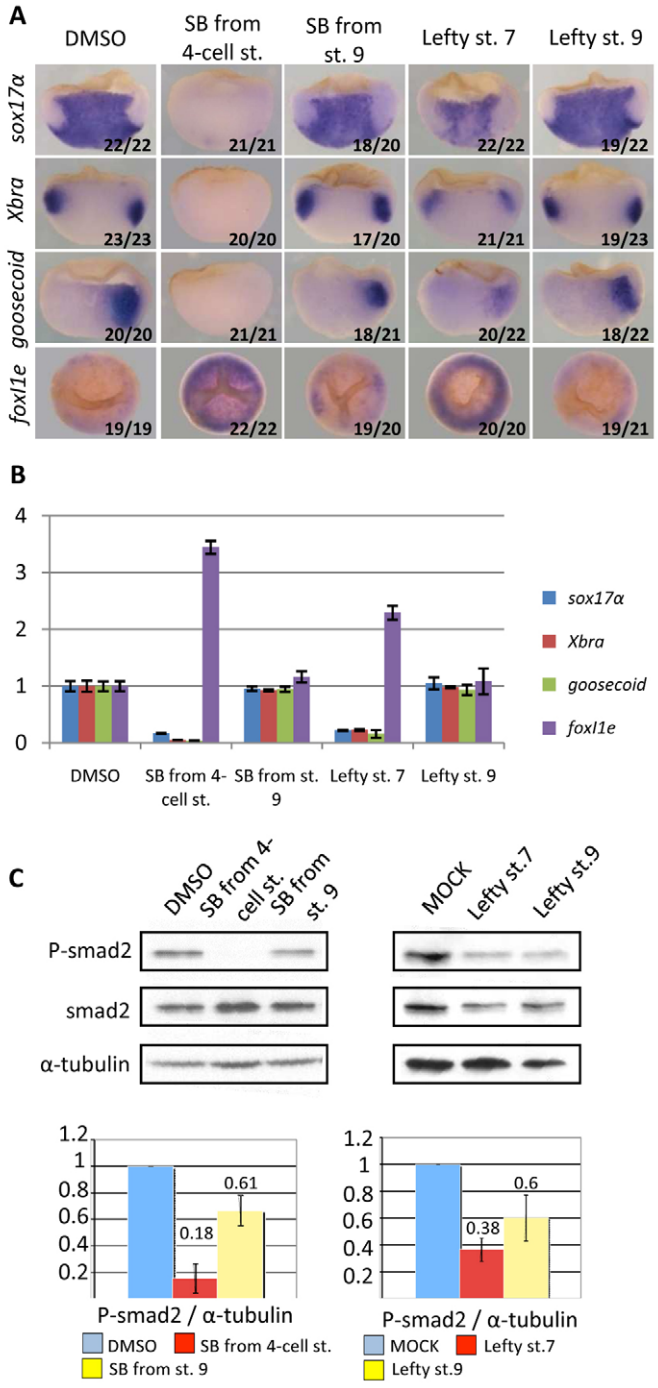

Fig. 1. Time-window of requirement for Nodal activity in mesendoderm induction. (**A**) *Xenopus* embryos were subjected to 200 μM SB-505124 (SB) or injected with 5 ng Lefty recombinant protein at the indicated stage (st.), and analysed by WISH at early gastrula stage. Control embryos were treated with DMSO. In this and the following figures, the number of embryos exemplified by the photograph over the total number of embryos analysed is indicated. For *sox17α*, *Xbra* and *goosecoid*, embryos were bisected prior to WISH and are viewed dorsal to the right, animal to the top. For *foxI1e*, views are animal, with dorsal to the top. (**B**) Q-PCR analysis was performed on the same embryos as in A. (**C**) Western blotting was performed on the same embryos as in A. The bar charts present the ratio of pSmad2 to α-tubulin levels, with respect to controls (DMSO and MOCK), which were set to 1.

S3C in the supplementary material). Such individuals also maintained contact-induced swimming behaviour, indicating that the neuromuscular system was functional (not shown). We conclude that Nodal signalling is necessary for gastrulation independently of its earlier role in mesendoderm induction.

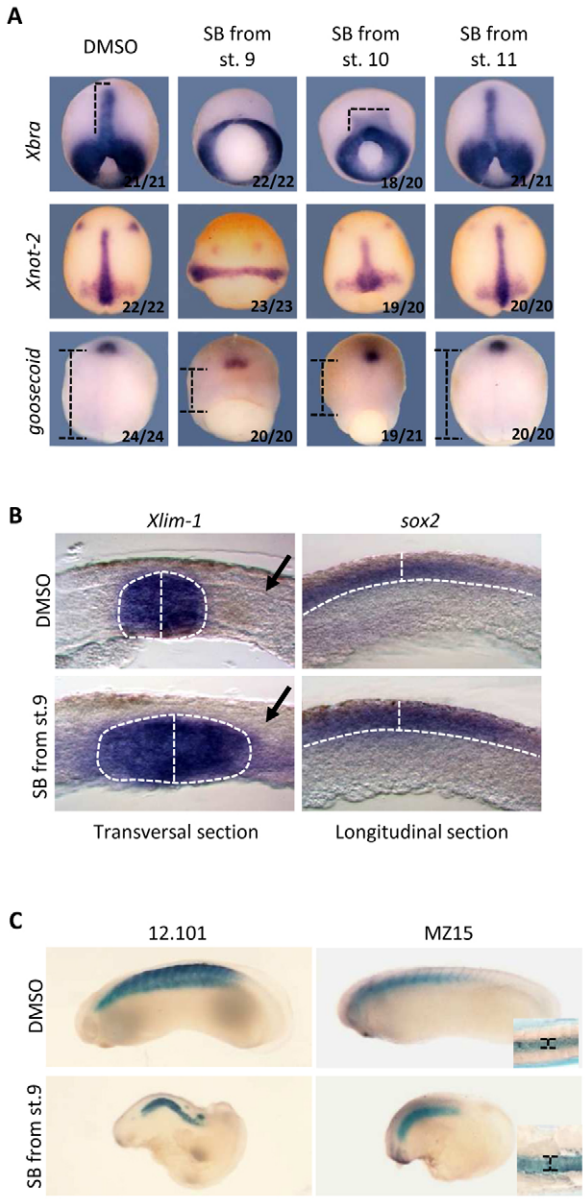

Fig. 2. Nodal signalling controls gastrulation independently of mesendoderm induction. (**A**) *Xenopus* embryos were subjected to 200 μM SB-505124 as indicated and analysed by WISH at early neurula stage. Notochord elongation, visualised by *Xbra* and *Xnot-2* staining (dashed lines), is reduced upon drug treatment prior to stage 11. The distance between the blastopore and the prechordal mesoderm expressing *goosecoid* (dashed line) is reduced in these embryos. (**B**) Late gastrula (stage 12) embryos were sectioned (50 μm) and stained for *Xlim-1* to reveal the extending notochord, and for *sox2* to mark the neural plate. The notochord was wider in drug-treated embryos, whereas the thickness of the neural tissue was unaffected. Note the absence of Brachet's cleft (arrows). (**C**) Immunostaining was used to reveal differentiated somitic muscles (12.101) and notochord (MZ15) at late tailbud stage. Horizontal sectioning, prior to staining, revealed the persistent reduction of convergent extension in notochordal tissue (insets).

Nodal signalling is necessary for convergent extension of dorsal mesoderm and for head mesoderm migration

Embryos lacking Nodal activity during gastrulation exhibited a distribution of notochordal tissue that was suggestive of defective convergent extension (Fig. 2A,B; see Fig. S3A,B in the supplementary material). To address this issue more directly, a red fluorescent dextran was unilaterally injected into axial progenitors at the 16-cell stage. At the end of gastrulation, the injected clone showed extensive rostrocaudal elongation in untreated embryos, but not in embryos treated with SB-505124 from stage 10 (Fig. 3A). This assay also allowed us to evaluate mediolateral intercalation, which is required for convergent extension. In the control condition, fluorescent and non-fluorescent cells intermingled at the border between the injected and non-injected territories (Fig. 3B). By contrast, no cell mixing occurred in embryos treated with SB-505124 from stage 10, indicating that cells did not intercalate in the mediolateral axis (Fig. 3B). Axial mesoderm cells also intercalate radially, i.e. in the vertical plane. We stained sagittally hemisected mid-gastrula embryos with phalloidin to evaluate radial intercalation. This mode of intercalation also appeared compromised in SB-505124-treated embryos, as more cell layers (5/6 instead of 3/4 in controls) were found in axial mesoderm, without an increase in the thickness of the tissue (Fig. 3C). The intercalation behaviour involves the adoption by mesoderm cells of a bipolar elongated shape, as revealed by phalloidin staining in the control condition (Fig. 3C). Cells did not elongate when subjected to SB-505124 at stage 10 (Fig. 3C). We conclude that mesoderm cell elongation and, consequently, intercalation behaviour, require proper Nodal signalling at the onset of gastrulation. Finally, we found that Brachet's cleft does not form in the absence of Nodal activity, suggesting that this pathway is important for tissue separation, a behaviour that is necessary for mesoderm migration on the blastocoele roof (Wacker et al., 2000) (Fig. 2B, Fig. 3C).

Next, we used dorsal marginal zone (DMZ) explants as another paradigm with which to evaluate the role of Nodal in convergent extension. In the control condition, DMZ taken at stage 10 and cultured for 12 hours showed extensive elongation. By contrast, DMZ cultured in the presence of SB-505124 or Lefty protein showed very little elongation and resembled control ventral marginal zone explants (Fig. 3D). We conclude that Nodal activity is important for convergent extension in both whole embryos and explants.

In Nodal-deficient embryos, the distance between the prechordal mesoderm, marked by *goosecoid*, and the blastopore was shorter than in the control (Fig. 2A). This might indicate reduced migration of rostral mesoderm. To evaluate this possibility, we used a spreading assay, whereby rostral mesoderm explanted at early gastrula stage is deposited on a fibronectin-coated slide (Nie and Chang, 2006). In the control condition, extensive spreading occurred after 3 hours of culture, revealing the normal migratory capacity of head mesoderm progenitors (Fig. 3E). In the presence of SB-505124, however, spreading was reduced to less than one-third, indicating that Nodal activity is required for prechordal mesoderm migration (Fig. 3E).

We conclude that Nodal signalling is required during gastrulation for convergent extension through its involvement in cell migratory and intercalation behaviour.

Xnr5 and Xnr6 act redundantly to induce mesendoderm

We showed above that uncoupling mesendoderm induction from gastrulation movements is possible with timely control of Nodal inhibition. We wanted to explore the possibility that these two

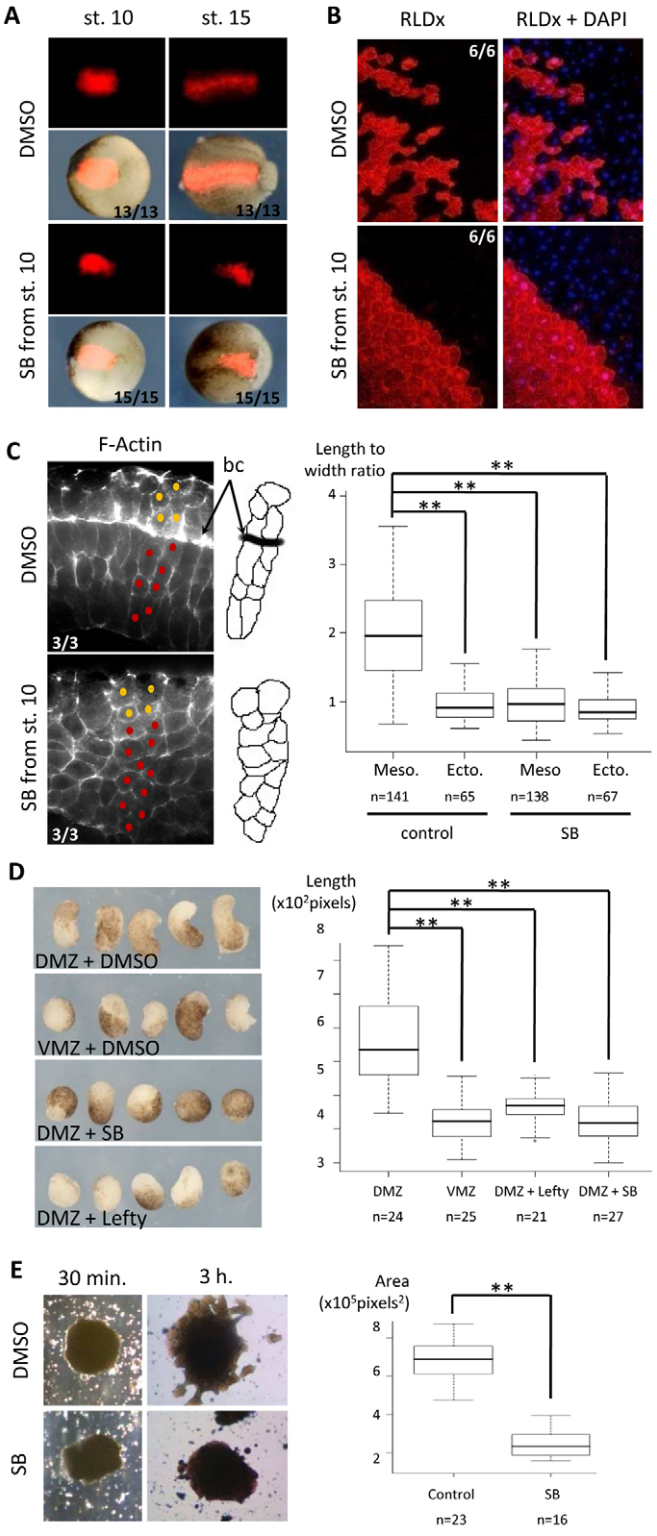

Fig. 3. Nodal signalling is required for convergent extension and head mesoderm migration. (**A**) *Xenopus* 16-cell embryos were injected with 2.5 ng rhodamine lysine dextran (RLDx) in one dorsal animal blastomere and cultured in DMSO or in 200 μM SB-505124 from stage 10. Embryos were photographed at stages 10 and 15 to reveal the initial and final distribution of the fluorescent clone. Dorsal views, anterior to the left. No rostrocaudal elongation of the injected clone occurred in Nodal-deficient embryos. (**B**) The axial mesoderm of stage 15 embryos in A was explanted and imaged from its internal face with an apotome microscope to reveal the distribution of fluorescent cells at the border of the injected clone. Anterior is at the top left corner. DAPI staining was used to reveal cell nuclei. Cells did not cross the border between injected and uninjected territories in the presence of the drug, indicating that intercalation was suppressed. (**C**) Embryos were cultured in DMSO or 200 μM SB-505124 from stage 10 to stage 12. Sagittal sections were stained with rhodamine phalloidin to reveal filamentous actin and cell shape. Axial mesoderm cells are indicated in red and neural ectoderm cells are indicated in yellow; anterior is to the left. Note the absence of Brachet's cleft (bc) in SB-505124-treated embryos. The total number of cell layers was increased in treated embryos (5 to 6 layers were present) compared with controls (3 to 4 layers present), indicating that radial (vertical) intercalation was reduced. The box plot shows the length (axis perpendicular to the surface of the ectoderm) to width (axis parallel to the surface of the ectoderm) ratio of both ectoderm and mesoderm cells. In this and all following box plots, the double asterisk indicates that the difference between two conditions is significant ($P<10^{-5}$, as calculated by the Kruskal-Wallis test). In the presence of SB-505124, mesoderm cells did not adopt their normal elongated shape. (**D**) Dorsal marginal zone (DMZ) was explanted at stage 10 and cultured for 12 hours in DMSO, in 50 μM SB-505124, or in the presence of 5 μg/ml Lefty protein. Ventral marginal zone (VMZ) explants served as non-elongating controls. The box plot shows the length of the explants. The absence of Nodal activity interfered significantly with DMZ elongation. (**E**) Head mesoderm was explanted at stage 10.25, plated on fibronectin-coated dishes, and cultured for 3 hours in the presence, or otherwise, of 50 μM SB-505124. Images were taken under visible light on an inverted microscope. The box plot shows the area of the explants after 3 hours of culture. The lack of Nodal activity in head mesoderm explants severely compromised spreading.

functions are controlled by distinct Nodal ligands expressed at different times. We reanalysed the expression of the best candidates, *Xnr1*, *Xnr2*, *Xnr5* and *Xnr6*, by Q-PCR between stage 6 (32 cell) and stage 10. We confirmed that the expression of *Xnr5* and *Xnr6* starts before the MBT, peaks when *Xnr1* and *Xnr2* transcripts start to accumulate soon after the MBT, and fades when *Xnr1* and *Xnr2* reach their maximum at stage 10 (see Fig. S4A in the supplementary material) (Takahashi et al., 2000; Yang et al., 2002). We thus decided to separately knockdown the Xnr5/Xnr6 and Xnr1/Xnr2 pairs of ligands with antisense morpholinos (MOs).

For each Xnr ligand we used two non-overlapping MOs designed to block translation: MOs of the 'a' series spanned the ATG, whereas MOs of the 'b' series targeted the 5′UTR. We first tested the ability of the 'a' series to inhibit the biological activity of Xnr mRNAs containing the targeted sequence. For this, we used an ectopic blastopore lip assay to assess the response to animal Xnr mRNA injection (Agius et al., 2000). We found that all MOs of the 'a' series could suppress ectopic blastopore lip formation by their cognate mRNA, including the multiple alleles of *Xnr5* (see Fig. S5 in the supplementary material) (Takahashi et al., 2006). In all subsequent analyses, knockdown by MOs of the two series yielded similar defects, demonstrating their specificity. As further confirmation, alterations in gene expression caused by the various knockdowns could be reversed by recombinant Nodal protein or Xnr mRNA injection, as detailed below.

When Xnr5 and Xnr6 MOs were injected separately, embryos developed normally and showed little reduction in mesendoderm gene expression (Fig. 4A,B; see Fig. S6 and Fig. S7A,B in the supplementary material). By contrast, double Xnr5/Xnr6 knockdown prevented gastrulation, and provoked a dramatic

repression of mesendoderm markers and a hyper-activation of *foxI1e* (Fig. 4A,B; see Fig. S6 and Fig. S7A,B in the supplementary material). The observed redundancy was not merely due to partial inhibition of each ligand, as increasing the amounts of MOs separately did not produce more severe defects, neither did the combination of Xnr5 MOa and MOb or of Xnr6 MOa and MOb (see Fig. S6 and Fig. S7B in the supplementary material). As expected, pSmad2 levels were more severely reduced upon double Xnr5/Xnr6 knockdown (82% reduction compared with control), than upon either single knockdown (46% reduction for Xnr5 MOa alone and 38% reduction for Xnr6 MOa alone) (Fig. 4C). Mesendoderm gene expression and pSmad2 levels were rescued to levels exceeding those of controls in response to Nodal protein injection in 'a', 'ab' and 'ba' Xnr5/Xnr6 morphants, and in response to the injection of a mixture of *Xnr5* and *Xnr6* mRNAs lacking the targeted sequences in 'b' morphants (Fig. 4A-C; see Fig. S6 and Fig. S7B in the supplementary material). WISH analysis showed that the expression of these markers was recovered in their normal territories, but was also expanded in ectoderm (Fig. 4A; see Fig. S6 in the supplementary material). We conclude that mesendoderm induction depends on the redundant activities of Xnr5 and Xnr6.

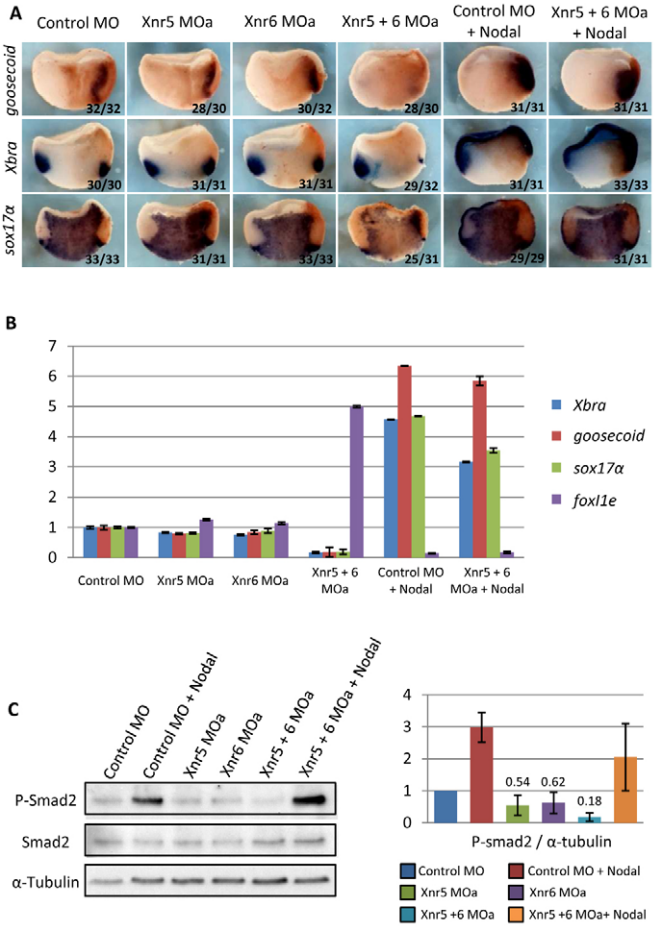

Fig. 4. Xnr5 and Xnr6 act redundantly to induce mesendoderm. (**A**) *Xenopus* 4-cell embryos were injected marginally in the two dorsal cells with 15 ng/cell of the indicated MOs, alone or in combination (7.5 ng each), along with 2.5 ng/cell fixable fluorescein lysine dextran (FLDx). For the purposes of rescue, 5 ng of Nodal recombinant protein was injected into the blastocoele at stage 8. Injected embryos were fixed at stage 10.25 and bisected prior to WISH. The presence of FLDx was revealed by immunostaining (orange). Embryos were photographed dorsal to the right (injected side), animal to the top. (**B**) Q-PCR analysis of embryos injected in all cells at the 4-cell stage with the same amounts of MO and/or Nodal as in A. (**C**) Western blotting was performed on the same embryos as in B.

Xnr1 and Xnr2 are required for gastrulation but play little role in mesendoderm induction

When Xnr1 and Xnr2 MOs were injected separately, embryos developed normally and showed unchanged levels of mesendoderm gene expression. By contrast, double Xnr1/Xnr2 knockdown prevented gastrulation in a permanent manner (Fig. 5A; see Fig. S8A,B,F in the supplementary material). In comparison to Xnr5/Xnr6 knockdown, mesendoderm gene expression did not collapse upon Xnr1/Xnr2 knockdown, but was only moderately downregulated, irrespective of the identity ('a' or 'b') and amounts of MOs used (see Fig. S8B-D in the supplementary material). As expected, pSmad2 levels were only partially reduced in Xnr1/Xnr2 morphants (35% reduction), comparable to the effect of SB-505124 inhibition started at stage 9 (see Fig. S8E in the supplementary material). Finally, late differentiation was apparent in the notochord and somitic mesoderm of double-morphant embryos (see Fig. S8F in the supplementary material). Thus, Xnr1 and Xnr2 are not the primary ligands controlling mesendoderm induction. They might, however, contribute to raising the total levels of Nodal activity above the threshold required for mesendoderm induction, as their expression was severely reduced in Xnr5/Xnr6 morphant embryos (see Fig. S7C in the supplementary material).

The Xnr1/Xnr2 double knockdown produced phenotypes similar to those caused by Nodal inhibition started at stage 9, as prechordal and chordamesoderm were present but abnormally positioned (Fig. 5A; see Fig. S8B in the supplementary material). We thus tested whether Xnr1/Xnr2 depletion provoked similar defective convergent extension and migration defects. It was apparent from the co-injection of a fluorescent dextran with Xnr1 and Xnr2 MOs that convergent extension did not occur normally (see Fig. S8A in the supplementary material). Furthermore, DMZ explants did not elongate upon double knockdown, whereas they did upon either single knockdown. Importantly, elongation was rescued in 'a' double-morphant explants exposed to Nodal recombinant protein and in 'b' double-morphant explants injected with a mixture of *Xnr1* and *Xnr2* mRNAs (Fig. 5B; see Fig. S8G in the supplementary material). Similar to Nodal chemical inhibition, Xnr1/Xnr2 knockdown by 'a' MOs suppressed head mesoderm migration on fibronectin, but spreading was rescued by exposure to recombinant Nodal (Fig. 5C). We conclude that Xnr1 and Xnr2 are crucial for gastrulation movements, whereas they play little role in mesendoderm induction.

Nodal signalling does not control gastrulation via the Wnt/PCP pathway

Non-canonical Wnt/PCP signalling is a prominent regulator of cell behaviours required for convergent extension. We thus addressed whether Nodal controls this pathway. We found that *Xwnt11*, the ligand responsible for PCP pathway activation, was still expressed in the presence of SB-505124 and Lefty. This is in agreement with the maintenance in such embryos of *Xbra*, which is known to directly activate *Xwnt11* (Fig. 6A,B) (Tada and Smith, 2000). As a control, we verified that FGFR inhibition by SU5402 repressed both genes (Fig. 6A,B). Similarly, *Fz7*, which encodes the receptor of Xwnt11, was expressed normally in the absence of Nodal activity (not shown). The first visible consequence of Wnt/PCP pathway activation is the recruitment of Dishevelled at the cell membrane

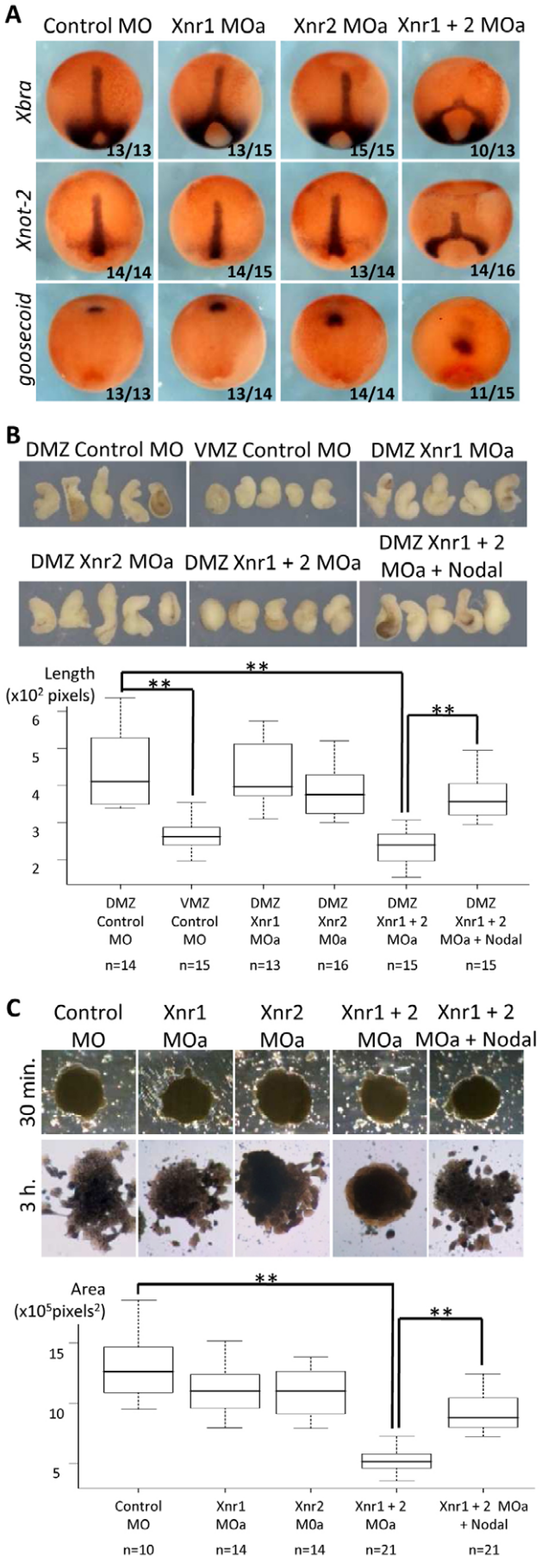

Fig. 5. Xnr1 and Xnr2 act redundantly to control gastrulation but not mesendoderm induction. (**A**) *Xenopus* 4-cell embryos were injected in the two dorsal cells with 5 ng/cell of the indicated MOs, alone or in combination (2.5 ng each), together with 2.5 ng/cell FLDx. Embryos were fixed at stage 15 and processed for WISH and FLDx staining. Dorsal views, anterior to the top. Extending notochord (*Xbra*, *Xnot-2*) and prechordal mesoderm (*goosecoid*) were present but abnormally positioned. (**B**) DMZ was explanted at stage 10 from the same embryos as in A and cultured for 12 hours. For the purposes of rescue, 5 μg/ml recombinant Nodal protein was added to the culture medium after explantation. Elongation was significantly suppressed in double Xnr1/Xnr2 morphant explants, but was rescued by Nodal protein. (**C**) Head mesoderm was explanted at stage 10.25 from the same embryos as in A, plated on fibronectin-coated dishes and cultured for 3 hours. Spreading was severely impaired in double Xnr1/Xnr2 morphant explants, but was rescued in the presence of 5 μg/ml recombinant Nodal protein.

(Wallingford et al., 2000). We co-expressed a Dsh-GFP fusion protein with a membrane-bound form of the fluorescent protein mCherry in the mesoderm, and treated early gastrula embryos with SB-505124 or SU5402. As expected, SU5402 prevented recruitment of Dsh-GFP at the membrane of mesoderm cells (Shi et al., 2009). By contrast, Dsh-GFP was present at the membrane of SB-505124-treated non-gastrulating embryos (Fig. 6C,D). Our data are consistent with the idea that the Wnt/PCP pathway remains largely functional in Nodal-deficient embryos.

Xnr1 and Xnr2 activate movement-effector gene expression

The data described above suggest that Nodal signalling directly controls the expression of non-Wnt/PCP movement-effector genes required for gastrulation. In order to test this, we selected several genes from the literature that are known to be involved in various cellular behaviours during gastrulation, including directed migration and adhesion. These genes encode the receptor tyrosine kinase PDGFRα (Nagel et al., 2004; Symes and Mercola, 1996), the small GTPase Rnd1 (Wunnenberg-Stapleton et al., 1999), the hyaluronan synthase HAS2 (Nardini et al., 2004), the protocadherin PAPC (Kim et al., 1998) and the putative N-acetyltransferase Camello (Popsueva et al., 2001). All these genes are expressed in invaginating mesendoderm and show dorsal enrichment at the onset of gastrulation (see Fig. S4B in the supplementary material), similar to *Xnr1* and *Xnr2* (Jones et al., 1995). WISH and Q-PCR analyses revealed that all these genes were severely downregulated when Nodal inhibition with SB-505124 or Lefty started at stage 9. Interestingly, all these genes were expressed at normal levels in FGFR-deficient embryos, highlighting the specificity of action of Nodal signalling (Fig. 7A; see Fig. S9A in the supplementary material). Similar to the trend observed for morphogenetic movements, the expression of all five genes was moderately reduced upon single knockdown, whereas it was severely repressed upon double Xnr1/Xnr2 knockdown (Fig. 7B; see Fig. S9B-D in the supplementary material). Importantly, injection of Nodal protein into the blastocoele at stage 9 rescued the expression of all markers in ‘a’, ‘ab’ and ‘ba’ Xnr1/Xnr2 morphants, whereas injection of a mixture of *Xnr1* and *Xnr2* mRNAs rescued ‘b’ double morphants. Again, expression was recovered in the normal territory, but was also expanded into the ectoderm, although differences between genes were observed (Fig. 7B; see Fig. S9B-D in the supplementary material).

We then addressed, using two complementary assays, whether Nodal could directly activate the expression of these genes. First, Nodal protein was injected into the blastocoele of late blastula embryos and expression measured by WISH (see Fig. S10 in the supplementary material). Second, animal caps explanted at the late blastula stage were exposed to Activin A and expression quantified by Q-PCR (Fig. 7C). In both setups, all genes were robustly activated in animal cells. To address whether this activation was direct, we combined Nodal or Activin A protein with CHX, a translation inhibitor. Both WISH and Q-PCR analyses revealed that all genes were still activated in the presence of CHX, except *rnd1*. As *rnd1* is known to be activated in a CHX-sensitive manner by Activin in animal caps (Ogata et al., 2007), this confirmed the efficiency of CHX in our assay.

From these analyses, we conclude that Nodal signalling mediated by Xnr1 and Xnr2 directly activates movement-effector genes during gastrulation, providing an initial explanation for the role of Nodal in morphogenesis that is independent of its role in mesendoderm induction.

DISCUSSION

Using three independent inactivation methods, we showed for the first time that Nodal signalling sequentially induces mesendoderm and controls gastrulation movements in a vertebrate embryo. In mouse, the unique Nodal ligand is required for mesendoderm specification, and an assessment of its role in movements will require conditional inactivation (Conlon et al., 1994). Time-dependent inhibition of Nodal signalling has also been performed in zebrafish with SB-431542 and SB-505124, although the process of gastrulation was not specifically addressed (Hagos and Dougan, 2007; Sun et al., 2006). Similar to our observations, these two studies showed that the period of mesendoderm induction controlled by Nodal signalling ends before the onset of gastrulation.

Our data illustrate the notion of division of labour, or sub-functionalisation, that is permitted by the expansion of gene families during evolution. This notion also applies to the zebrafish family of Nodal ligands, as Cyclops and Squint (Nodal-related 2 and Nodal-related 1, respectively – Zebrafish Information Network) act redundantly to induce mesendoderm (Feldman et al., 1998), whereas Southpaw is expressed during somitogenesis and controls left-right patterning (Long et al., 2003). Intriguingly, strategies for sub-functionalisation evolved independently, as in *Xenopus* it is Xnr1 that controls left-right patterning (Toyoizumi et al., 2005), although it is also required for gastrulation together with Xnr2 (this work).

We propose that Nodal ligands control two zygotic transcriptional programmes that are initiated at different times (Fig. 8). *Xnr5* and *Xnr6* are the first zygotic genes to be activated by the crucial maternal regulators of frog embryonic development, VegT and β-catenin (Takahashi et al., 2000; Yang et al., 2002). Prior to stage 9, these two ligands together activate a mesendodermal genetic programme that primarily includes regulators such as Sox17α, Xbra, Goosecoid and Xlim-1. Importantly, Xnr1 and Xnr2 are also part of this programme as they can be activated by Xnr5 and Xnr6 (Takahashi et al., 2000) and their expression depends on them (this work). After stage 9, and until the mid-gastrula stage, Xnr1 and Xnr2 become the main canonical Nodal ligands in endomesoderm and activate a movement genetic programme comprising effector proteins that are directly involved in the cell behaviours of gastrulation (Fig. 8). A role for the divergent Xnr3 in convergent extension has also been reported, but it appears to mobilise the FGF/Xbra cassette and is thus unlikely to contribute to the programme uncovered in this work (Yokota et al., 2003). The mechanism(s) regulating the transition between the two Nodal-dependent programmes remains unclear. We can envision two complementary scenarios. The first is based on a differential promoter sensitivity to Nodal/Smad2 of genes in the two programmes, whereby higher levels of Nodal activity are required to activate genes of the morphogenesis programme. Such levels would be reached only once Xnr1 and Xnr2 start to signal, further raising the levels of pSmad2 induced by Xnr5 and Xnr6. Supporting this idea, Xnr1/Xnr2 knockdown or late blastula inhibition by SB-505124 provokes a more modest reduction of pSmad2 levels than Xnr5/Xnr6 knockdown or early inhibition by the drug, and represses movement genes but not mesendoderm regulators. The second

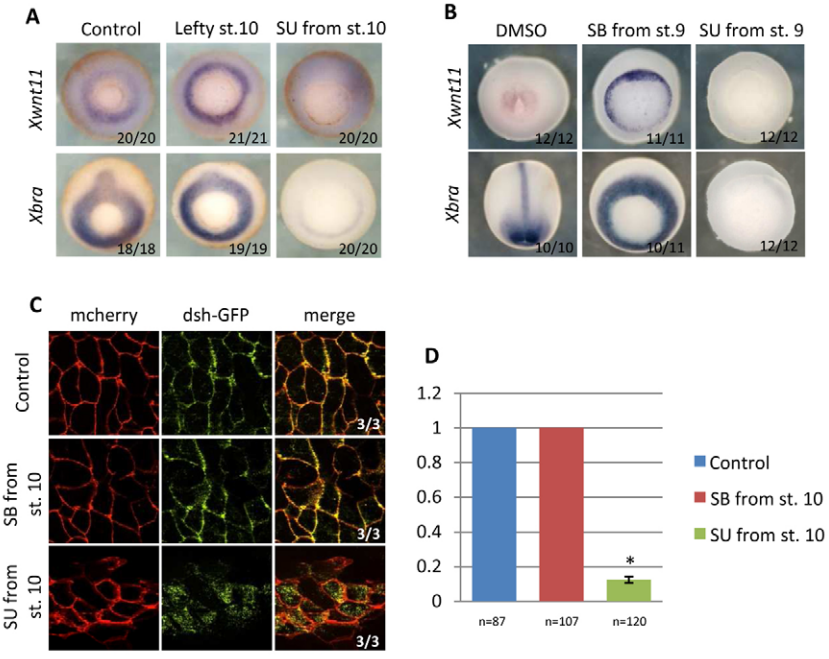

Fig. 6. The Nodal pathway does not regulate the Wnt/PCP pathway. (**A**,**B**) *Xenopus* embryos were injected with 10 ng Lefty recombinant protein, treated with 200 μM SU5402 or 200 μM SB-505124 at the indicated stages, and analysed by WISH at stage 12 (A) or 14 (B). SU5402, but not SB-505124, repressed *Xbra* and *Xwnt11*. All views are vegetal. (**C**) Four-cell embryos were marginally injected with a mixture of 250 pg/blastomere *dsh-GFP* and 1 ng/blastomere *mcherry* mRNAs in the two dorsal cells. Stage 12 embryos were fixed, sectioned, stained with an anti-GFP antibody and imaged with an apotome microscope. (**D**) The percentage of cells that displayed Dsh-GFP fluorescence predominantly at the cell membrane among the mCherry-positive cells. Three embryos were analysed per condition, and the total number of cells analysed is indicated. *, P=0.0314.

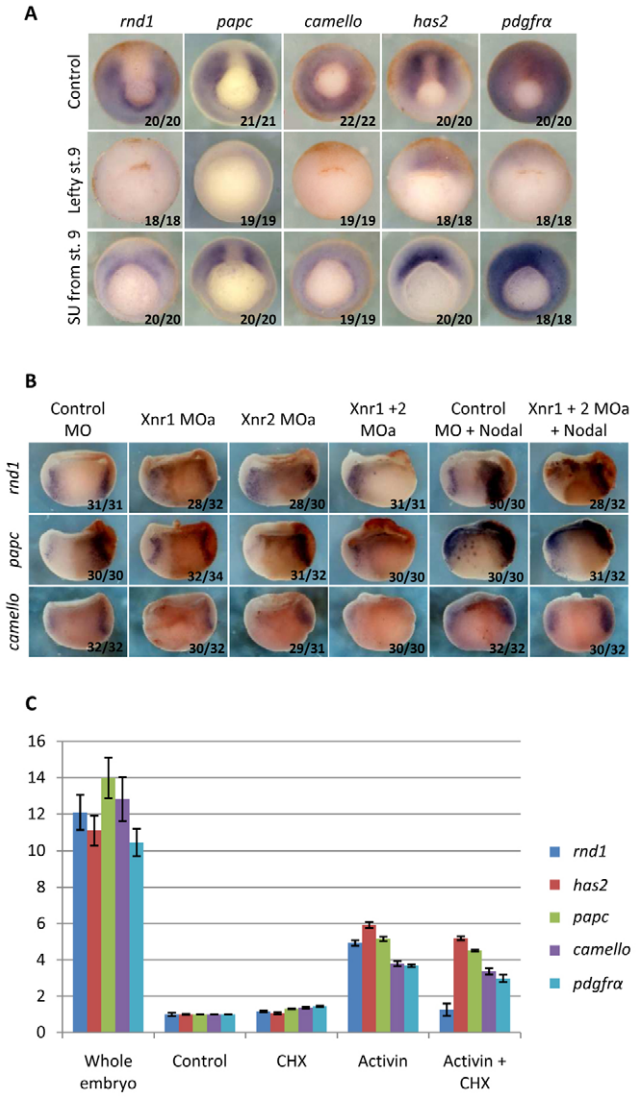

Fig. 7. Nodal signalling directly activates movement-effector genes. (**A**) *Xenopus* embryos were injected with 10 ng Lefty recombinant protein or treated with 200 µM SU5402 at stage 9, and analysed by WISH at stage 12. Vegetal views, dorsal to the top. (**B**) Four-cell embryos were injected in the two dorsal cells with 15 ng/cell of the indicated MOs, alone or in combination (7.5 ng each), together with 2.5 ng/cell FLDx. For the purposes of rescue, 5 ng of Nodal recombinant protein was injected into the blastocoele at stage 9. Injected embryos were fixed at stage 10.25 and bisected prior to WISH. Dorsal to the right (injected side), animal to the top. Movement-effector gene expression was severely downregulated in Xnr1/Xnr2 double-morphant embryos, but was rescued by the reintroduction of Nodal protein. (**C**) Animal caps were prepared at stage 8.5, preincubated in the presence of CHX for 1 hour, exposed to 5 ng/ml Activin A and CHX for another 2 hours, and then harvested and processed for real-time RT-PCR. Nodal activated all genes in a CHX-independent manner, except *rnd1*, a known indirect target of Activin signalling.

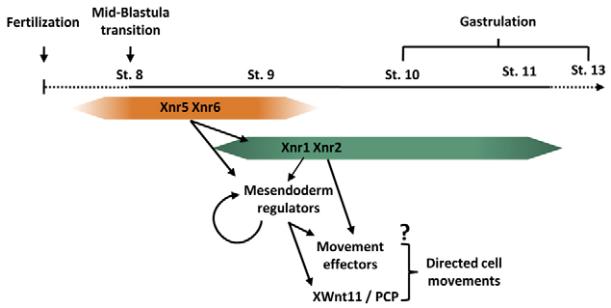

Fig. 8. Working model for the control of gastrulation by Nodal and Wnt/PCP. Xnr5 and Xnr6 activate the mesendoderm programme, as well as *Xnr1* and *Xnr2*, which in turn activate movement effectors. Xnr1 and Xnr2 may also contribute to the activation of the mesendoderm programme. This first programme is made up of regulators (Xbra, Goosecoid, Sox17, Xlim-1 and many others) that are capable of interacting in a self-sustaining network that does require Nodal for its maintenance. The mesendoderm regulators also contribute to the activation of Nodal-dependent movement effectors and the Wnt/PCP pathway. Directed cell movements during gastrulation might ultimately depend on the polarised distribution or activity of Nodal-regulated effectors by the Wnt/PCP pathway.

mechanism involves the regulation of movement genes by both Nodal signalling and mesendoderm regulators (Fig. 8). Consistent with this idea, movement gene expression is only partially suppressed in Xnr1/Xnr2 morphants or in embryos treated with Nodal inhibitors from stage 9. This view is also supported by evidence in the literature, including the reported regulation of *papc* expression by Xlim-1 (Hukriede et al., 2003) or of *Xwnt11* by Xbra (Tada and Smith, 2000). This mechanism would be analogous to the feedforward loops that help deploy the early mesoderm gene regulatory network in the fly (Sandmann et al., 2007). The two mechanisms proposed here would ensure that movements occur only once fates have been specified. The full validation of our model awaits genome-wide identification of the promoters targeted by Nodal/Smad2 before and during gastrulation.

We have shown that the Nodal pathway controls the migration of prechordal mesoderm as well as convergent extension through mediolateral and radial intercalation in chordamesoderm, a behaviour known to be controlled by the Wnt/PCP pathway (Solnica-Krezel, 2005; Wallingford et al., 2002). Interestingly, we found no evidence for regulation of the Wnt/PCP pathway by Nodal during gastrulation. This suggests that Nodal might function in parallel to the Wnt/PCP pathway. An attractive hypothesis would be that the Nodal pathway activates the expression of effectors that are necessary for movements, whereas the Wnt/PCP pathway polarises the distribution or activity of these effectors to provide directionality (Fig. 8). The next challenge will be to determine which particular cellular properties and effectors are regulated by Nodal in gastrulating tissues.

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Competing interests statement
The authors declare no competing financial interests.

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