New roles for FoxH1 in patterning the early embryo

Matt Kofron[1], Helbert Puck[1], Henrietta Standley[1], Chris Wylie[1], Robert Old[2], Malcolm Whitman[3] and Janet Heasman[1,*]

[1]Division of Developmental Biology, Cincinnati Children's Research Foundation, 3333 Burnet Avenue, Cincinnati, OH 45229, USA
[2]Biomolecular Medicine Group, Department of Biological Sciences, University of Warwick, Gibbet Hill Road, Coventry CV4 7AL, UK
[3]Department of Cell Biology, Harvard Medical School, Boston, MA 02115, USA
*Author for correspondence (e-mail: heabq9@chmcc.org)

Accepted 5 August 2004

Development 131, 5065-5078
Published by The Company of Biologists 2004
doi:10.1242/dev.01396

Summary

FoxH1 (Fast1) was first characterized as the transcriptional partner for Smad proteins. Together with Smad2/4, it forms the activin response factor (ARF) that binds to the *Mix.2* promoter in *Xenopus* embryos. *Foxh1* is expressed maternally in *Xenopus*. Depletion of maternal *Foxh1* mRNA results in abnormalities of head and dorsal axis formation. We show that FoxH1 is required, together with XTcf3/β catenin, to activate the zygotic expression of the nodal gene, *Xnr3* in a Smad2-independent manner. In contrast, maternal FoxH1 acts as an inhibitor of Xnr5 and 6 transcription, preventing their upregulation on the ventral side of the embryo, by the maternal T-box transcription factor VegT. We conclude that maternal FoxH1 has essential, context-dependent roles in regulating the pattern of zygotic gene expression in the early embryo.

Key words: *Foxh1*, *Fast1*, Antisense, Nodal, *Xnr5*, *Xnr6*, *Xnr3*, *Xenopus*

Introduction

FoxH1 (Fast1) was first characterized as the transcriptional partner for Smad proteins forming the activin response factor (ARF) binding to the *Mix.2* promoter in *Xenopus* embryos (Chen et al., 1996). *Foxh1* family members have been described in many vertebrate groups (for a review, see Carlsson and Mahlapuu, 2002). They show high homology in the fork-head DNA binding and Smad interaction domains and very little conservation outside those domains. Mice lacking FoxH1 are embryonic lethal and show defects ranging from total lack of embryonic structures, lack of anterior structures, or less severe notochord and node defects (Hoodless et al., 2001; Yamamoto et al., 2001). Analyses of these phenotypes concluded that FoxH1 was the major transcriptional transducer of nodal signaling in early development (Yamamoto et al., 2001). In contrast, zebrafish maternal/zygotic mutants of *Foxh1* (*schmalspur*) had less severe phenotypes consisting of cyclopia, loss of floorplate and posterior chordal plate and ventral body curvature (Pogoda et al., 2000; Sirotkin et al., 2000). Also *schmalspur* mutants were able to induce the expression of the organizer gene *goosecoid* in response to nodal signaling, suggesting that FoxH1 is not strictly required to transmit nodal signals in zebrafish (Pogoda et al., 2000). However, studies on double mutants of zebrafish *Foxh1* and *Mix*-like gene (*Bon*/*Sur* mutants) also placed zFoxH1 in the nodal signaling pathway (Kunwar et al., 2003; Trinh et al., 2003).

In *Xenopus*, experiments with activator and repressor constructs, and blocking antibodies, suggested that FoxH1 activates mesendodermal gene expression and controls gastrulation movements (Watanabe and Whitman, 1999). Loss of function experiments using a morpholino approach had a less extreme effect on development, but also indicated a potential role for FoxH1 in gastrulation movements, since the convergence extension movements of activin-induced animal caps were blocked by FoxH1 depletion (Howell et al., 2002). A second early zygotic member of the Fox family, Fast3, was also found to be expressed specifically during the gastrula stage. Loss of function experiments on Fast3 showed similar phenotypes to those caused by morpholino-induced depletion of FoxH1 (Howell et al., 2002). FoxH1 has been shown to bind to DNA in the absence of activated Smads and interacts relatively weakly with activated Smads compared to Fast3 (Howell et al., 2002), raising the possibility that maternal FoxH1 may have other, Smad-independent functions.

Since controversy remains on the relative requirement for FoxH1 in pattern formation and nodal signal transduction in vertebrates, we have specifically analysed the contribution of maternal *Foxh1* in *Xenopus* early development using an antisense oligo-mediated approach. This approach has been useful in demonstrating that the maternal T-box transcription factor VegT is necessary and sufficient for the establishment for both mesodermal and endodermal germ layers (Zhang et al., 1998), and that the cytoplasmic protein β catenin establishes the dorsal axis by relieving the repressive effects of the HMG box transcription factor XTcf3, on target genes such as *goosecoid* (Houston et al., 2002).

Here we depleted *Foxh1* mRNA from stage 6 oocytes using an antisense oligonucleotide and assayed the effect on development. We show that maternal FoxH1-depleted embryos are headless and lack axial structures. FoxH1 depletion results in a severe inhibition of the activation of a FoxH1 reporter ARE-luciferase. Even so, nodal responsiveness is not lost in animal caps, and mes-endodermal gene expression continues

in FoxH1-depleted embryos. We find that the expression of the organizer gene *Xnr3*, which is a direct target of the maternal Wnt signaling pathway, is most sensitive to FoxH1 depletion. Using *Foxh1*/*XTcf3* double depletions, we show that FoxH1 is required, together with XTcf3 de-repression by β-catenin, to activate *Xnr3* expression in a Smad2-independent fashion. In contrast, we find that maternal FoxH1 inhibits the ectopic expression of *Xnr5* and *6* in the ventral vegetal area of the late blastula. We conclude that FoxH1 is required to regulate the spatio-temporal patterns of *Xnr3*, *5* and *6* expression.

Materials and methods

Oocytes and embryos

Full-grown oocytes were manually defolliculated and cultured in oocyte culture medium (OCM), as described previously (Xanthos et al., 2001). Oocytes were injected in their vegetal hemispheres with oligo using a Medical Systems picoinjector and oocytes were cultured a total of 48-72 hours at 18°C before fertilization with a sperm suspension. In preparation for fertilization, they were stimulated to mature by the addition of 2 μM progesterone to the culture medium and cultured for 10-12 hours. Oocytes were then labeled with vital dyes and introduced into stimulated female hosts using the host-transfer technique described previously (Zuck et al., 1998). Embryos were maintained in 0.1×MMR, and all the colored, experimental embryos were sorted from host embryos. Unfertilized eggs and abnormally cleaving embryos were removed from all batches. For explant experiments, control and FoxH1-depleted embryos were dissected into the parts shown at the mid-blastula stage in 1×MMR on 2% agarose dishes, and cultured in OCM until the early gastrula stage before freezing. For dorsal/ventral explants, the dorsal sides of embryos were marked with crystals of Nile Blue sulfate at the four-cell stage and bisected on 2% agarose dishes at the late blastula or early gastrula stage as described in the text.

Fixation and histology

For histology and X-gal staining, embryos were fixed in MEMFA for 2 hours, rinsed in PBS and stained using X-gal. For histology, embryos were dehydrated, embedded in low-melt wax, serially sectioned at 20 μm and stained with Haematoxylin and Eosin.

In situ hybridization

Embryos for in situ hybridizations for *Xnr5* and *Xnr6* were prepared by fixing whole blastulae for 1 hour in MEMFA, bisecting the embryos along the dorsal-ventral axis with a scalpel blade, fixing for one additional hour in MEMFA, washing and storing in 100% ethanol. The in situ hybridizations were performed as described (Harland, 1991) using BM Purple as substrate (Roche) with two exceptions. The RNase A/T1 digestion was omitted from the protocol and the anti-digoxigenin antibody was diluted in MAB-blocking buffer then pre-absorbed with embryonic acetone powder before embryo incubation.

Oligos and mRNAs

The antisense FoxH1 oligo used was an 18-mer 5′-C*A*G*CTTCATCGCATC*C*A*G-3′ where * indicates a phosphorothioate bond, and other linkages were phosphorodiester bonds. The oligo was resuspended in sterile, filtered water and was injected in doses of 2.5-5 ng per oocyte. The oligos for depletion of VegT (5′-C*A*G*CAGCATGTACTT*G*G*C-3′) and XTcf3 (5′-C*G*A*G*GGATCCCAGTC*T*T*G*G-3′) were used as described previously (Houston et al., 2002; Zhang et al., 1998). The oocytes were cultured immediately at 18°C. *Foxh1* mRNA was synthesized by linearizing the plasmid vector pCS2+FoxH1 with *Not*I, and transcribing the linear template with SP6 polymerase in the presence of cap analog and GTP using the Megascript kit (Ambion). RNA was ethanol precipitated and resuspended in sterile, distilled water for injection.

Western blot analysis

Western blot analysis with anti-phospho-Smad2 antibody was used after affinity purification from crude antisera (Peter ten Dijke), and using secondary goat anti-rabbit IgG-HRP antibody (Boehringer Mannheim). Western analysis was carried out as described by Lee et al. (Lee et al., 2001).

Luciferase assay

The firefly luciferase reporter construct pGL3-ARE-luciferase, consisting of three repeats of the activin response element (ARE) containing the FoxH1 binding sites from the regulatory sequence of the *Mix.2* gene was used as described previously (Huang et al., 1995). It was injected into specific cells of the early embryo as described in the text, in doses of 50 pg, together with 10 pg of control HSTK *Renilla luciferase* plasmid. Pools of four or five embryos or five animal caps were collected in triplicate for each injection mixture at stage 10. Luciferase assays were performed using the Dual Luciferase Reporter Assay system (Promega). Five embryos were homogenized in 100 μl of lysis buffer, and cleared by microcentrifugation. The supernatant (20 μl) was assayed in 50 μl of assay mixture, and luciferase activity was measured for 10 seconds with an analytical luminescence laboratory monolight 2010. Firefly luciferase activity was normalized to *Renilla* activity. Each experiment was repeated at least twice, and single representative experiments are shown.

Analysis of gene expression using real-time RT-PCR

Total RNA was prepared from oocytes, embryos and explants using proteinase K and then treated with RNase-free DNase as described (Zhang et al., 1998). Approximately 1/6 embryo equivalent of RNA was used for cDNA synthesis with oligo (dT) primers followed by real-time RT-PCR and quantitation using the LightCycler System (Roche) as described in Kofron et al. (Kofron et al., 2001). The primers and cycling conditions used are listed in Table 1. Relative expression values were calculated by comparison to a standard curve generated by serial dilution of uninjected control cDNA. Samples were normalized to levels of ornithine decarboxylase (ODC), which was used as a loading control. Samples of water alone, or controls lacking reverse transcriptase in the cDNA synthesis reaction, failed to give specific products in all cases. Experiments were repeated at least twice on different oocyte and embryo batches to ensure that the pattern of gene expression described was reproducible from one experiment to the next.

Results

The depletion of maternal FoxH1

We targeted maternal *Foxh1* mRNA by injecting phosphorothioate/diester antisense oligonucleotides into full-grown *Xenopus* oocytes. The half-life of such oligos is less than 12 hours, and injected oocytes were cultured for at least 48 hours before fertilization, ensuring that the oligos and target RNA were degraded and allowing protein turnover. Of 20 oligos tested, oligo10 depleted *Foxh1* mRNA to 10-20% of the control level (Fig. 1A). To examine the degree to which *Foxh1* mRNA levels were affected during embryogenesis, a staged series of control and oligo10-injected embryos were frozen and analysed by real-time PCR. We confirmed that, in control embryos, *Foxh1* expression is highest in the oocyte, and gradually reduces through gastrulation (Fig. 1A) (Chen et al., 1996; Howell et al., 2002). In FoxH1-depleted embryos, the level of *Foxh1* mRNA does not recover after the mid-blastula transition, but continues to decline through the gastrula and

Table 1. PCR primer pairs and PCR cycling conditions used with the Lightcycler™

PCR primer pair	Origin	Sequence	Denaturing temperature (°C)	Annealing temperature (°C)/time (sec)	Extension temperature (°C)/time (sec)	Acquisition temperature (°C)/time (sec)
Chordin	XMMR*	U: 5′-AACTGCCAGGACTGGATGGT-3′ D: 5′-GGCAGGATTTAGAGTTGCTTC-3′	95	55/5	72/12	81/3
Cerberus	Heasman et al., 2000	U: 5′-GCTTGCAAAACCTTGCCCTT-3′ D: 5′-CTGATGGAACAGAGATCTTG-3′	95	60/5	72/20	81/3
dikkopf	New	U: 5′-CACCAAGCACAGGAGGAA-3′ D: 5′-TCAGGGAAGACCAGAGCA-3′	95	56/5	72/10	82/3
Fast3	New	U: 5′-AACCCCCAGAGCTGAAGAAC-3′ D: 5′-TCATATTGGCCCCTCATAGC-3′	95	56/5	72/14	88/3
Fgf8	Kofron et al., 1999	U: 5′-CTGGTGACCGACCAACTAAG-3′ D: 5′-ACCAGCCTTCGTACTTGACA-3′	95	55/5	72/14	86/3
Foxh1	New	U: 5′-TTCTACAATCTCCCCCGTTG -3′ D: 5′-AGAAGTGGGCAAGTCCAAT -3′	95	56/5	72/12	81/3
Goosecoid v.2	New	U: 5′-TTCACCGATGAACAACTGGA-3′ D: 5′-TTCCACTTTTGGGCATTTTC-3′	95	55/5	72/11	82/3
GS17	XMMR*	U: 5′-ATGCCAGTCCAACTTCAAGGCA-3′ D: 5′-CCTCTAGCATAGATGGACTGTA-3′	95	60/5	72/20	81/3
Mix.2	New	U: 5′-TGATGGACTATGAATGGAGTGAA-3′ D: 5′-CCCCAAACTGTGGTGTTACC-3′	95	55/5	72/10	81/3
Mixer	Xanthos et al., 2001	U: 5′-CACCAGCCCAGCACTTAACC-3′ D: 5′-CAATGTCACATCAACTGAAG-3′	95	55/5	72/12	83/3
Odc	Heasman et al., 2000	U: 5′-GCCATTGTGAAGACTCTCTCCATTC-3′ D: 5′-TTCGGGTGATTCCTTGCCAC-3′	95	55/5	72/12	83/3
Siamois	Heasman et al., 2000	U: 5′-CTGTCCTACAAGAGACTCTG-3′ D: 5′-TGTTGACTGCAGACTGTTGA-3′	95	55/5	72/16	81/3
Xbra	Sun et al., 1999	U: 5′-TTCTGAAGGTGAGCATGTCG-3′ D: 5′-GTTTGACTTTGCTAAAAGAGACAGG-3′	95	55/5	72/8	75/3
Xlim1	Kofron et al., 2004	U: 5′-CCCTGGCAGCAACTATGACT-3′ D: 5′-GGTTGCCATAACCTCCATTG-3′	95	55/5	72/11	85/3
Xnr1 v.2†	New	U: 5′-AGAGGAATGTGGGTGCAGTT-3′ D: 5′-CAACAAAGCCAAGGCATAAC-3′	95	55/5	72/10	76/3
Xnr2 v.2†	New	U: 5′-TTACTGTATGAAGACGGAGAAGTTG-3′ D: 5′-ATGCGACATGCCACAAAAC-3′	95	55/5	72/10	77/3
Xnr3 v.2†	New	U: 5′-TAATCTGTTGTGCCGATCCA-3′ D: 5′-ATCAATGTTGCCCTTTTTCA-3′	95	56/5	72/9	79/3
Xnr5 v.2†	New	U: 5′-TGGTTGGGGAGACCTGATTA-3′ D: 5′-AGAGGCCTCATCTTCACTGG-3′	95	55/5	72/10	77/3
Xnr6 v.2†	New	U: 5′-AAGATTGGATGGGGTCATCA-3′ D: 5′-ATCAGCATGGACAAGGGACT-3′	95	55/5	72/10	80/3
Xvent2	New	U: 5′-TGAGACTTGGGCACTGTCTG-3′ D: 5′-CCTCTGTTGAATGGCTTGCT-3′	95	62/5	72/20	83/3
Xsox17α	Xanthos et al., 2001	U: 5′-GCAAGATGCTTGGCAAGTCG-3′ D: 5′-GCTGAAGTTCTCTAGACACA-3′	95	58/5	72/8	85/3

*XMMR, *Xenopus* Molecular Marker Resource (http://www.xenbase.org/xmmr/Marker_pages/primers.html).
†*v.2*, new version of primer designed with primer3 web based program (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi).

neurula stages. This indicates that the maternal store of *Foxh1* mRNA is not supplemented or replaced by zygotic transcripts. In contrast, the related gene, *Fast3*, is not expressed in oocytes, and begins to be expressed after the mid-blastula transition (MBT; Fig. 1B). The timing of MBT is indicated by the onset of expression of the zygotic transcript GS-17 (Fig. 1B).

Since the only available antibody is not sensitive enough to detect endogenous FoxH1 (data not shown), we confirmed that the activity of FoxH1 protein was reduced by testing the ability of a FoxH1-reporter construct (ARE-luciferase) (Watanabe and Whitman, 1999) to respond to activin in wild-type and FoxH1-depleted animal caps. Fig. 1C shows that there is a significant reduction of activity of the luciferase reporter in FoxH1-depleted caps at the early gastrula stage compared to the levels induced by activin in control caps.

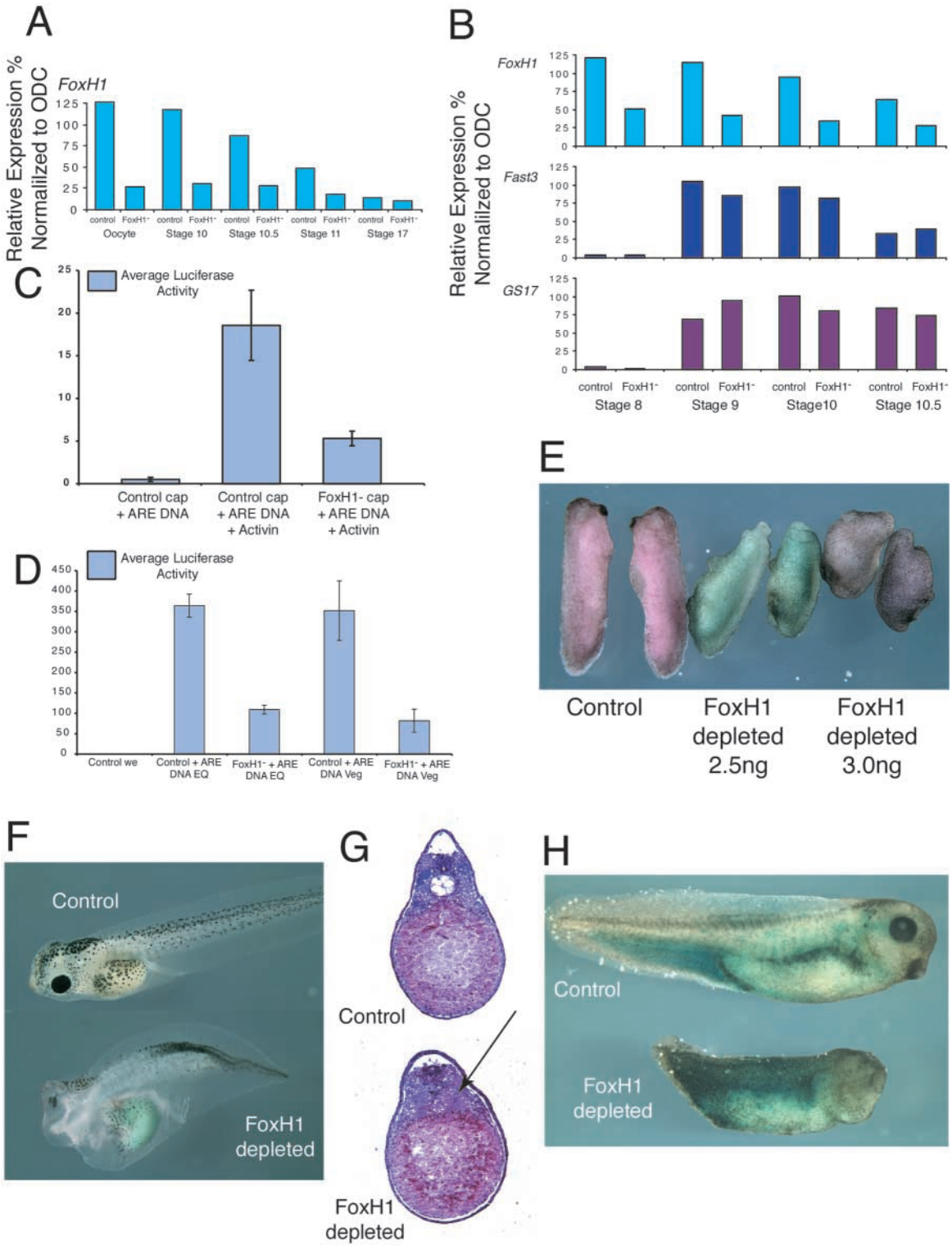
To test the effect of FoxH1 depletion on the activation of the ARE-luciferase by endogenous TGFβ signaling in the early embryo, we injected ARE-luciferase into the vegetal area, or equatorial region of control or FoxH1-depleted embryos at the four-cell stage, and analyzed the level of induction of luciferase activity at the early gastrula stage. Fig. 1D shows that the ARE-luciferase is activated to similar levels when injected equatorially or vegetally into control embryos, and this activation is significantly reduced in depleted embryos.

These results show that the loss of FoxH1 is sufficient to severely reduce the activity of the FoxH1-reporter construct in response to both exogenous and endogenous ARE-inducing signals.

Maternal FoxH1 is required for head formation

To examine the effects on morphogenesis of FoxH1-depletion, we injected the antisense oligo into defolliculated oocytes, cultured them for 48 hours, to allow the target mRNA and the injected oligo to degrade the mRNA, and then matured and

fertilized the eggs by the host-transfer technique (Zuck et al., 1998). FoxH1-depleted embryos developed normally through gastrulation but axial defects became apparent during neurulation and were obvious at the tailbud and tadpole stages (Fig. 1E,F). In seven experiments, 108 of 114 control uninjected embryos developed normally, while only four, FoxH1-depleted embryos were normal and 109 were headless or had more extreme axial deficiencies, including a dose-dependent shortening of the axis as shown in Fig. 1E. In histological sections taken at the tailbud stage of headless embryos, three germ layers were visible, but axial structures were abnormal. In particular, the notochord was absent or reduced (5/5 cases examined) and somites fused across the midline (arrow in Fig. 1G). While initial gut formation appeared normal, gut looping was disrupted. Heart tissue developed, but was also abnormal (Fig. 1F). Using real-time



RT-PCR analysis of late neurula stage embryos, we confirmed that mesodermal and endodermal tissues were specified. MyoD (somite marker) and Xsox17 (endoderm marker) were relatively normally expressed, whereas anterior endodermal (Pdx1) and heart marker (Nkx2.5) expression was reduced (data not shown). Although the formation of anterior structures was most impaired, the anterior-posterior axis was not altered, since the β-galactosidase-labeled progeny of ventral cells injected at the four-cell stage were found predominantly in the posterior and trunk of both control and FoxH1-depleted embryos (Fig. 1H and data not shown).

Maternal FoxH1 regulates Xnr3

Since this phenotype strongly resembled that caused by partially blocking the maternal Wnt signaling pathway by depleting maternal β catenin (Heasman et al., 1994), we first confirmed that maternal *β catenin* mRNA levels were unaffected in FoxH1-depleted embryos (data not shown). Next we examined the expression in FoxH1-depleted embryos of the known targets of the maternal XTcf3/β catenin signaling pathway, including *Xnr3*, *siamois*, *goosecoid* and *chordin*. FoxH1 depletion resulted in a loss of the expression of the organizer gene, *Xnr3* (reduced to less than 10% in 6/6 experiments). Expression of other organizer genes, particularly *chordin*, was also reduced but was not as dramatically or consistently affected as *Xnr3*. We confirmed that these changes were not simply due to a delayed onset of expression, by comparing the expression patterns at 2-hour intervals over an 8-hour period, during which *Xnr3* expression peaks and falls in control, uninjected embryos (Fig. 2A). *Xnr3* expression did not reach wild-type levels in FoxH1-depleted embryos at any stage during gastrulation. This shows that FoxH1 is required for the activation of *β catenin*/*XTcf3* target genes, and that *Xnr3* is most sensitive to its depletion.

To confirm that these effects were specifically caused by the reduction of maternal *Foxh1* mRNA, we determined the appropriate dose of mRNA for rescue experiments. When *Foxh1* mRNA was over-expressed in oocytes in doses greater than 100 pg, embryos developed with a headless phenotype (data not shown). We therefore tested the effect of *Foxh1* mRNA over-expression on the activity of the ARE-luciferase reporter: 15-120 pg *Foxh1* mRNA was injected into the vegetal area of two-cell stage embryos that had been injected with ARE-luciferase at the one-cell stage, and luciferase activity was examined at the early gastrula stage. Fig. 2B indicates that 15 pg *Foxh1* mRNA activates the ARE-reporter to a higher level than the level caused by endogenous signals, while higher doses repress ARE-luciferase activity, compared to control levels. In a second experiment, 30 pg *Foxh1* mRNA activated luciferase, while higher doses caused inhibition (data not shown). Thus, FoxH1 acts as an activator or repressor of the ARE-luciferase reporter in a concentration-dependent manner.

For rescue experiments, oligo 10-injected and control oocytes were incubated for 48 hours (to allow oligo and mRNA degradation) and then 15 and 30 pg *Foxh1* mRNA was injected in the vegetal area. Oocytes were matured and fertilized and allowed to develop to the tailbud stage. Siblings were frozen at the gastrula stage for the analysis of molecular markers of axis formation. *Foxh1* mRNA significantly rescued the expression of *Xnr3*, and other organizer genes, in a dose-responsive fashion (Fig. 2C); 30 pg also significantly rescued head formation in 80% of embryos (8/10 cases) compared to sibling FoxH1-depleted embryos which had 100%-reduced heads or headless phenotype (15/15 cases; Fig. 2D). The experiment was repeated with a similar result. These results indicate that the embryo is extremely sensitive to the level of expression of *Foxh1* mRNA, and confirms that FoxH1 regulates the expression of *Xnr3*, and head formation.

In previous studies we have shown that the expression of *Xnr3* is regulated by maternal β catenin, which blocks the repression of *Xnr3* expression by the maternal HMG box protein XTcf3 (Houston et al., 2002). We next tested whether there was genetic interaction between XTcf3 and FoxH1, by partially depleting maternal stores of *XTcf3* and *Foxh1* mRNAs, both singly and together. Fig. 3A shows that while partial FoxH1 depletion alone (2.5 ng oligo) caused a reduction of *Xnr3* expression, double-depleted embryos lose *Xnr3* expression completely. This experiment was repeated three times with the same result. The organizer gene, *goosecoid*, has been shown to be a target of FoxH1 in zebrafish (Sirotkin et al., 2000). In comparison to *Xnr3*, *goosecoid* was reduced but not eliminated in XTcf3/FoxH1^{-} embryos (Fig. 3A). Double-depleted XTcf3/FoxH1^{-} embryos had more severe axial defects, than those caused by the depletion of XTcf3 or FoxH1 alone (Fig. 3B).These results show that the wild-type level of *Xnr3* expression requires the combinatorial activity of maternal FoxH1 transcriptional activation together with XTcf3 de-repression by β catenin.

Maternal FoxH1 and the nodal signaling pathway

FoxH1 was first characterized as the transcriptional partner for

Fig. 1. Antisense depletion of maternal FoxH1: 4 ng FoxH1 antisense oligo injected into oocytes causes a depletion of *FoxH1* mRNA in oocytes that is maintained through early embryogenesis. (A,B) Control and FoxH1-depleted oocytes and embryos derived from the same batch of oocytes were cultured to the stages shown, frozen, and assayed by real-time RT-PCR. Expression levels were normalized to ODC. (A) No wave of zygotic transcription of *Foxh1* is seen in control or FoxH1-depleted embryos at the gastrula and neurula stages. (B) The related family member Fast3 is a zygotic transcript, expressed in control and FoxH1-depleted embryos, at the same time as the marker of the mid-blastula transition, GS17. Oocytes and embryos were cultured, frozen, and assayed by real-time RT-PCR. Expression levels were normalized to ornithine decarboxylase (ODC). (C) ARE-luciferase activity is induced in animal caps by activin protein (10 ng/ml), and this induction is severely inhibited in FoxH1-depleted animal caps at the early gastrula stage. (D) ARE-luciferase activity is induced by endogenous nodal signaling in the vegetal (DNA Veg), or equatorial region (DNA EQ) of control embryos at the early gastrula stage. This activation is significantly reduced in FoxH1-depleted explants. (E) FoxH1 antisense oligo causes dose-responsive effects on head and axis formation. Oocytes injected with 2.5 and 3 ng of oligo develop as embryos with a headless phenotype. (F) The morphology of a FoxH1-depleted embryo at the swimming tadpole stage compared to control. (G) In histological sections, headless, FoxH1-depleted embryos at the late tailbud stage embryos have abnormal dorsal axes, lacking notochords and with somites fused across the midline (arrow). (H) Tailbud stage wild-type and FoxH1 embryos showing Xgal labeled progeny of one ventral cell injected at the four-cell stage, at the equator. Although FoxH1 depletion reduces the length of the embryo, the progeny of the ventral cell are in the same posterior and trunk locations as the control.

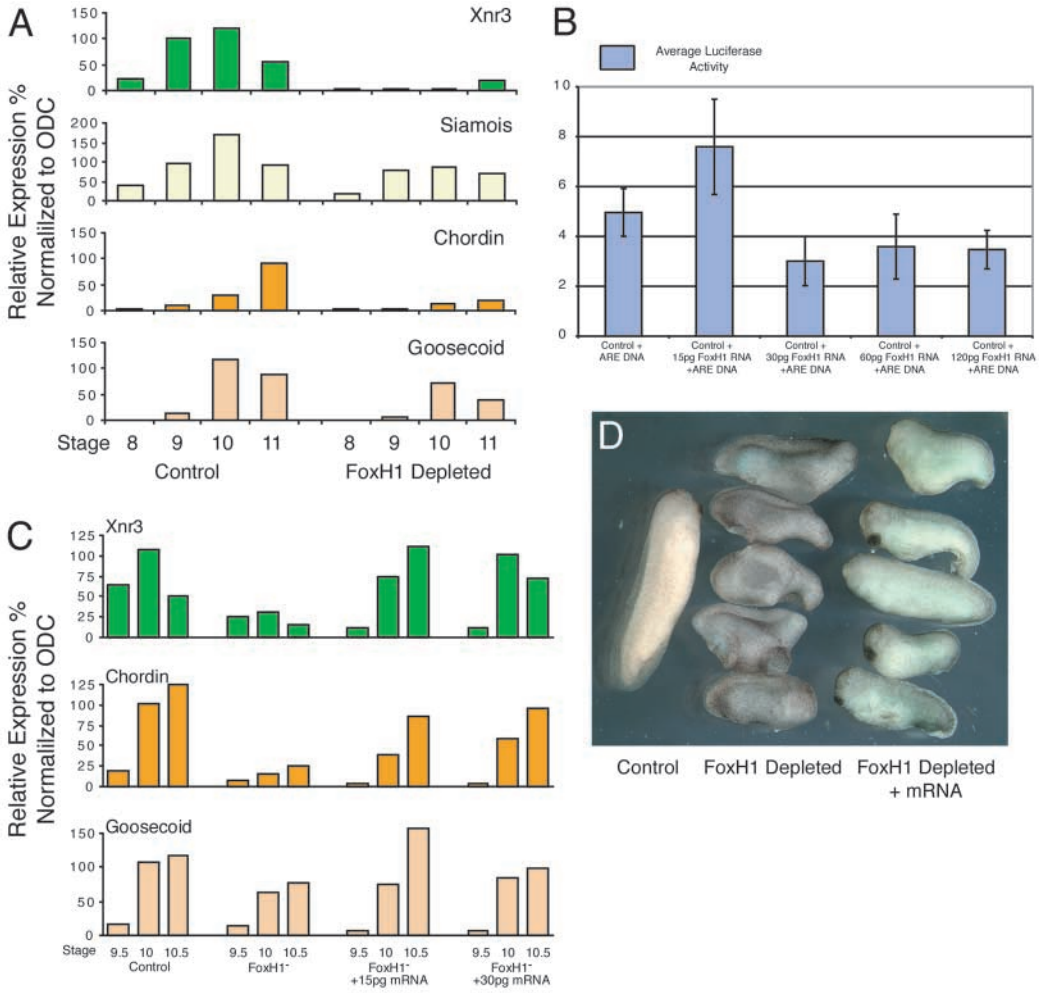


Fig. 2. FoxH1 regulates *Xnr3* gene expression in a specific fashion. (A) Embryos derived from oligo injected and control uninjected oocytes were frozen at 2-hour intervals during the blastula and gastrula stages, and assayed by real-time RT-PCR for the relative expression of organizer genes *Xnr3*, *siamois*, *chordin* and *goosecoid*. Expression levels were normalized to ODC. (B) 15 pg of *Foxh1* mRNA injected into the vegetal area of wild-type embryos causes increased activation of ARE-luciferase reporter compared to control levels, while higher doses inhibit the activation of ARE-luciferase reporter. (C) The reintroduction of 15 or 30 pg of *Foxh1* mRNA into FoxH1-depleted oocytes before maturation rescues the expression of *Xnr3* and *chordin* mRNA at the early gastrula stage. (D) The reintroduction of 30 pg of *Foxh1* mRNA into FoxH1-depleted oocytes before maturation rescues the headless phenotype.

phospho-Smad2, and one important question is whether its regulation of Xnr3 expression requires Smad2. Previous experiments have shown that Xnr3 continues to be expressed in maternal VegT-depleted embryos in which nodal signaling is prevented and in which Smad2 phosphorylation has been demonstrated to be blocked (Lee et al., 2001; Xanthos et al., 2002), suggesting that Xnr3 expression is activated by FoxH1 independently of Smad2 phosphorylation. To confirm this, we blocked nodal signaling by injecting 500 pg mRNA encoding the nodal-specific binding form of Cerberus, CerS into wild-type and FoxH1-depleted oocytes (Agius et al., 2000). Fig. 3C shows that *Xnr3* expression was reduced, to 60% of control levels, by *CerS* mRNA injection into wild-type embryos. In comparison, *Xnr1* expression, which is known to be dependent on phospho-Smad2 signaling, was reduced to 15% of control levels. We showed by Western blot using a phospho-Smad2-specific antibody (Fig. 3D) that introducing 500 pg *CerS* mRNA into oocytes and fertilizing them, completely blocked Smad2 phosphorylation in embryos at the gastrula stage. Taken together, these results show that maternal FoxH1 regulation of *Xnr3* is not strictly Smad2 dependent.

The nodal signaling pathway has been shown in mouse, zebrafish and *Xenopus* embryos to be important for head and trunk mesoderm formation, as well as the establishment of the endoderm germ layer (reviewed in Whitman, 2001; Schier, 2003). FoxH1 is considered to be a major effector of the nodal signaling pathway (reviewed by Osada et al., 2000; Schier, 2003; Whitman, 2001), although several studies have also reported FoxH1-independent nodal signaling routes (Germain et al., 2000; Ohkawara et al., 2004; Sirotkin et al., 2000). Fig.1C,D showed that FoxH1 depletion severely reduced FoxH1-reporter activity in response to activin or to endogenous inducing signals. To examine the extent to which loss of maternal FoxH1 affected the embryo's response to TGFβ signals, we dissected wild-type or FoxH1-depleted animal caps at the mid-blastula stage and treated them with activin (10

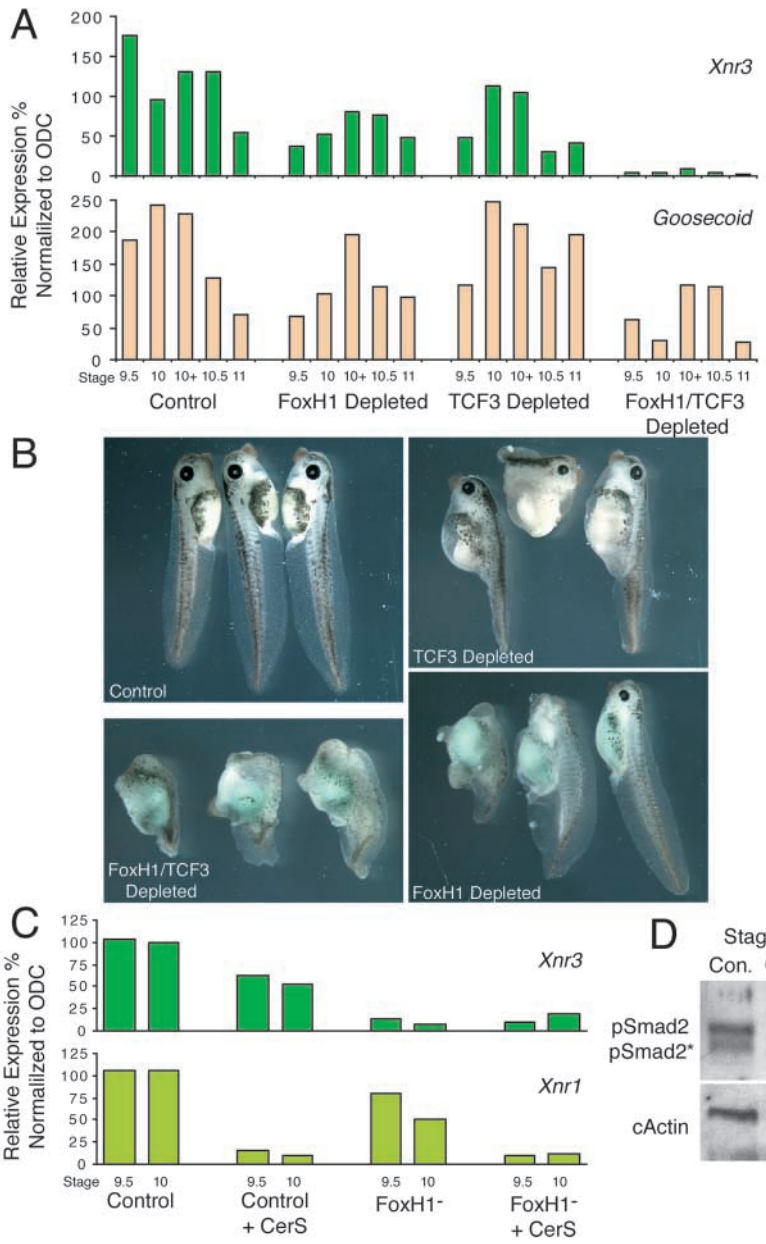
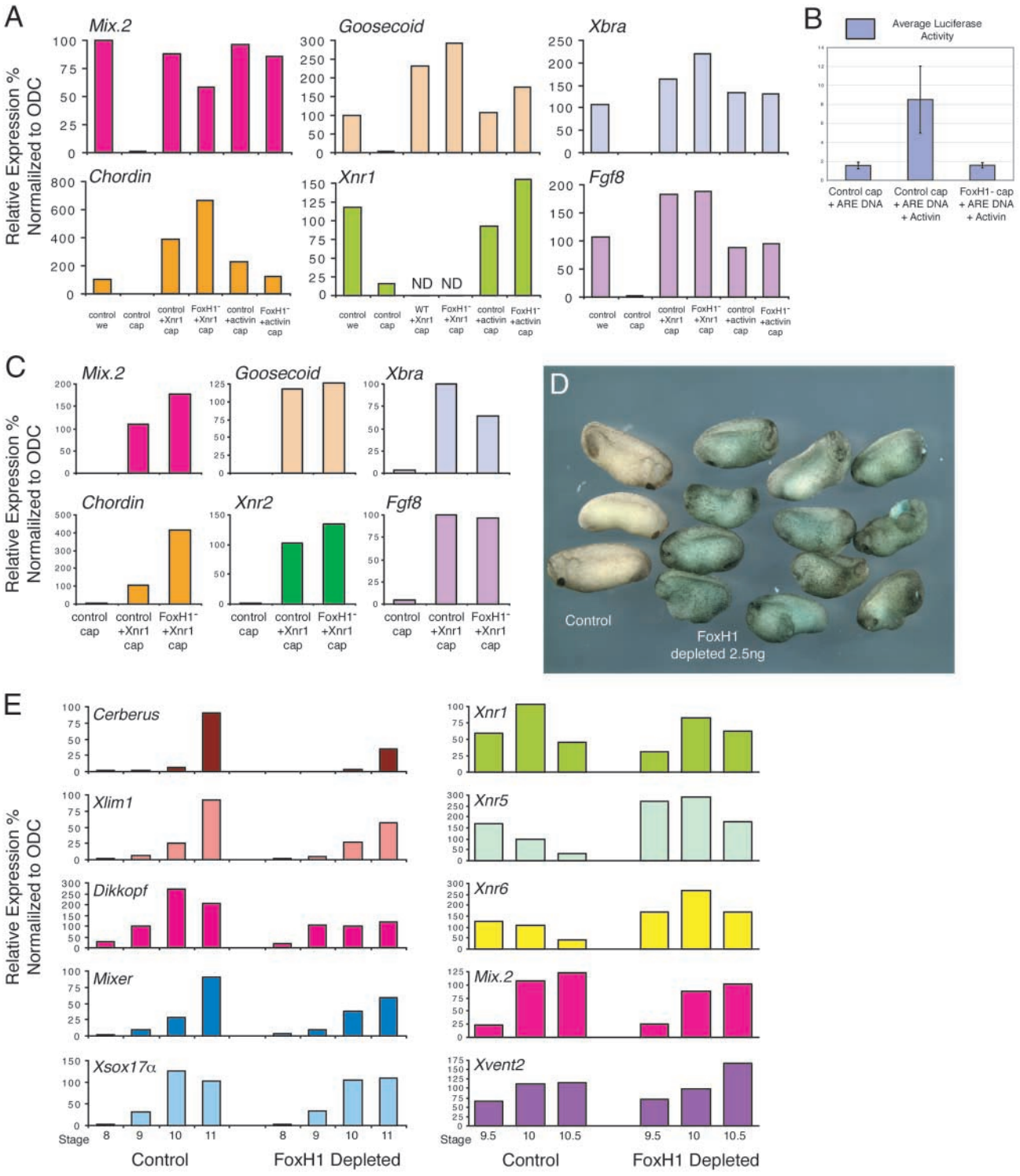


Fig. 3. Partial depletion of maternal XTcf3 and FoxH1 together causes a complete loss of *Xnr3* expression. (A) Oocytes were injected with 6 ng of XTcf3 oligo, 2.5 ng of FoxH1 oligo or both, cultured for 58 hours and then fertilized by the host transfer method. Embryos derived from these oocytes were frozen at two-hourly intervals through the late blastula and gastrula stages and analysed by real-time RT-PCR for the expression of *Xnr3* and *goosecoid* mRNAs. Double-depleted embryos have complete loss of expression of *Xnr3* mRNA throughout the gastrula stages. In comparison, *goosecoid* mRNA was reduced but not eliminated in double-depleted embryos. (B) Sibling embryos to those analyzed in (A) were allowed to develop to the tailbud stage. Phenotypically, partially XTcf3-depleted embryos have an anteriorized phenotype (TCF3 depleted), Partial FoxH1-depleted embryos have reduced or absent anterior structures and double-depleted embryos have more severe axial defects, than those caused by the depletion of XTcf3 or FoxH1 alone. (C) Nodal signaling was inhibited in control and FoxH1-depleted oocytes by the injection of 500 pg of *CerS* mRNA into oocytes. Embryos were frozen at the late blastula and early gastrula stages and assayed for *Xnr3* and *Xnr1* mRNA expression. *Xnr3* continues to be expressed in control embryos in which nodal signaling is blocked by CerS. In contrast, *Xnr1* expression is dependent on nodal signaling. (D) Western blot analysis with anti-phospho-Smad2 (pSmad2) antibody of wild-type embryos and embryos injected as oocytes with 500 pg *CerS* mRNA and analysed at two stages during gastrulation. CerS blocks nodal signaling and completely prevents Smad2 phosphorylation (as well as Smad2* phosphorylation; a second phosphorylated form). The blot was reprobed for actin as a loading control.

μg/ml) for 4 hours before assaying for gene expression at the mid-gastrula stage. Alternatively, we injected *Xnr1* mRNA (2 pg), into wild-type or FoxH1-depleted embryos at the two-cell stage, dissected caps at the mid-blastula stage and analysed them at the mid-gastrula stage for the expression of genes normally upregulated by TGFβ signaling (*Mix.2*, *Xnr1*, *Fgf8*, *Xbra*, *goosecoid* and *chordin*; Fig. 4A). Since depletion of maternal FoxH1 substantially reduced the FoxH1 binding construct, ARE-luciferase, from responding to activin, we expected that FoxH1 depletion would cause a reduction in the induction of nodal response genes in this assay. Surprisingly, *Mix.2*, *Xnr1*, *Fgf8*, *Xbra*, *goosecoid* and *chordin* were induced normally in maternal FoxH1-depleted animal caps treated with activin protein or *Xnr1* mRNA (Fig. 4A), and sibling caps cultured to the tail-bud stage elongated to similar extents as controls (data not shown). To confirm this result, we tested, within one experiment, the degree of reduction of ARE-luciferase activity, and the level of induction of nodal-response genes in sibling animal caps and correlated these with the phenotype of sibling embryos at the tailbud stage. Fig. 4B-D confirmed that FoxH1 depletion severely limited the ability of activin to activate the ARE, returning luciferase levels to the control, non-induced state, and causing a headless phenotype. However it did not prevent Xnr1 inducing *Mix.2*, *chordin*, *Fgf8*, *Xbra* and *goosecoid* in animal caps. This demonstrates that the induction of nodal response genes in animal caps by Xnr1 and activin does not depend on maternal FoxH1 or the formation of ARF. These zygotic genes are not only 'nodal response genes' but are activated by other pathways as well.

Since FoxH1-depleted embryos have obvious abnormalities of head formation, we next surveyed the endogenous level of expression of early zygotic mesodermal and endodermal genes activated by VegT in *Xenopus* (Kofron et al., 1999; Xanthos et al., 2001; Xanthos et al., 2002), in a temporal series of wild-

type and maternal FoxH1-depleted embryos, for other genes that were clearly regulated by FoxH1 (Fig. 4E). All the endodermal and mesodermal genes studied, (*cerberus*, *Dkk*, *Xlim1*, *Xbra*, *Mix.2*, *Xnr1* and *Mixer*) were reduced by FoxH1 depletion compared with controls, but none showed the extreme depletion of *Xnr3*, even though sibling embryos were headless at the tail-bud stage. In contrast, the nodal family members *Xnr5* and *6* were upregulated by the loss of maternal FoxH1 (Fig. 4E). The experiment was repeated eight times, and although some variation was seen in individual marker expression, the trends were consistent. Since the depletion of FoxH1 in these experiments is incomplete, and the effect on *cerberus*, *Dkk*, *Xlim1*, *Xbra*, *Mix.2*, *Xnr1* and *Mixer* expression is only partial, we cannot distinguish whether their remaining expression is due to the remaining FoxH1, or to other regulatory pathways. These results show, however, that FoxH1 is required to downregulate *Xnr5* and *6* expression, and to modulate the levels of expression of many mes-endodermal genes.



FoxH1 inhibits the expression of *Xnr5* and *6* mRNA in the ventral vegetal region of the blastula

Since the upregulation of *Xnr5* and *6* mRNAs by FoxH1 depletion was unexpected, we first showed that this effect could be rescued by the injection of 15 or 30pg of *Foxh1* mRNA into FoxH1 depleted oocytes (Fig. 5A). Next we examined when and where the over-expression of *Xnr5* mRNA caused by FoxH1 depletion occurred. Fig. 5B shows that the expression of *Xnr5* mRNA is detected at the mid-blastula stage in both control and FoxH1-depleted embryos and is enhanced in FoxH1-depleted embryos two hours later, at the late blastula stage. To confirm that FoxH1 depletion caused increased expression of Xnr5 and 6, we carried out in situ hybridization on hemisected embryos at the late blastula stage. Xnr5 and 6 are not abundant mRNAs and are difficult to detect by in situ (Takahashi et al., 2000). Their expression is vegetally localized and nuclear at the late blastula stage and is enhanced by FoxH1 depletion (Fig. 5C). Next we examined the expression of *Xnr5* and *6* in animal, equatorial and vegetal explants (Fig. 5D), and also in dorsal and ventral half embryos at the late blastula and early gastrula stages (Fig. 5E). We found that maternal FoxH1 depletion caused an increased expression of *Xnr5* and *6* in the vegetal mass, specifically on the ventral side (arrows in E). The experiment was repeated twice with the same result. This shows that maternal FoxH1 normally prevents the ectopic expression of *Xnr5* and *6* mRNA in ventral vegetal cells.

Next we asked whether *Xnr5* and *6* regulation by FoxH1 required nodal-signaling. We examined the expression of *Xnr5* and *6* in wild-type and FoxH1-depleted embryos injected before maturation with 500 pg *CerS* mRNA. Fig. 5F shows that, unlike *Xnr1* mRNA, which is much reduced by blocking nodal signaling (Fig. 3C), *Xnr5* and *6* mRNA expression is enhanced by blocking nodal signaling with *CerS* in control embryos. Again, depleting FoxH1 enhanced *Xnr5* expression but there was no additive effect of inhibiting both nodal signaling with CerS and depleting FoxH1. This suggests that the inhibition of *Xnr5* and *6* expression by FoxH1 is nodal signaling-dependent.

Fig. 4. FoxH1 depletion does not prevent animal caps from responding to activin or Xnr1. (A) Groups of ten control or FoxH1-depleted animal caps were dissected at the mid-blastula stage, treated with activin (1 μg/ml) for 4 hours and frozen at the early gastrula stage and assayed for expression of nodal target genes including *Mix.2*, *Fgf8*, *Xbra*, *goosecoid*, *chordin*, *Xbra* and *Xnr1*. In the same experiment, *Xnr1* mRNA (2 pg), was injected into wild-type or FoxH1-depleted embryos at the two-cell stage, and caps dissected as above. Expression levels were compared with one wild-type embryo at the early gastrula stage (control we). No significant changes in activin and nodal target gene expression were seen in FoxH1-depleted caps compared to control caps. (B,C,D) In one experiment, the degree of reduction of ARE-luciferase activity (B), and the level of induction of nodal-response genes in sibling animal caps (C) was measured at the gastrula stage, and the phenotype of sibling embryos was examined (D) at the tailbud stage. (B) FoxH1-depleted caps are unable to activate ARE-luciferase in response to activin (1 μg/ml). (C) Sibling FoxH1-depleted caps respond normally to *Xnr1* mRNA (2 pg) injected at the two-cell stage by expressing *Mix.2*, *chordin*, *Fgf8*, *Xbra*, *Xnr2* and *goosecoid* as measured by real time RT-PCR. (D) Sibling embryos (right) develop with a headless phenotype compared to controls (three embryos on the left). (E) The expression of mesodermal and endodermal early zygotic genes analysed by real-time RT-PCR in a temporal series of control and FoxH1-depleted embryos, frozen at the blastula and gastrula stages. All of these results were repeated in a second experiment. Most endodermal and mesodermal genes show some reduction of expression. *Xnr5* and *6* showed increased expression compared to control levels.

Maternal FoxH1, XTcf3 and VegT regulate *Xnr5* and *6* expression

Previous studies have shown that *Xnr5* and *6* expression is repressed in the early embryo by maternal XTcf3, and that their activation requires both β catenin and VegT (Hilton et al., 2003; Xanthos et al., 2002). We therefore asked whether removing both maternal XTcf3 and FoxH1 would act in an additive fashion to enhance *Xnr5* expression. This was not the case. In two experiments, depletion of either FoxH1 or XTcf3 enhanced the expression of *Xnr5*, but the double depletion did not increase its expression further (Fig. 6A).

Xnr5 expression has been shown previously to be activated by the maternal transcription factor VegT (Hilton et al., 2003; Takahashi et al., 2000; Xanthos et al., 2002). To examine whether the ectopic expression of *Xnr5* seen in FoxH1-depleted embryos is also dependent on VegT activity, we compared the levels of expression of *Xnr5* in embryos depleted of both maternal VegT and FoxH1. Fig. 6B shows that the increased expression of *Xnr5* caused by FoxH1 depletion is completely lost in the absence of VegT, indicating that the ectopic expression of *Xnr5* in FoxH1-depleted embryos is dependent on VegT transcriptional activation. In comparison, *Xnr3* expression is not prevented by VegT depletion, confirming that it is not regulated by VegT. Taken together, these results show that *Xnr3* and *5* are regulated differently by FoxH1. *Xnr3* is activated in a VegT/nodal independent fashion, and *Xnr5* is inhibited in a VegT/nodal dependent fashion.

The mis-regulation of *Xnr3* and *Xnr5* mRNA in FoxH1 depleted gastrulae contributes to their abnormal development at the tailbud stage

Since Xnrs are known to be potent signaling molecules, we reasoned that the downregulation of *Xnr3* and upregulation of *Xnr5* and *6* mRNA may be responsible for the later abnormal development of *FoxH1*-depleted embryos.

In previous studies, we and others have shown that the depletion and over-expression of *Xnr3* mRNA results in embryos that have reduced heads and abnormalities in convergence extension movements (Smith et al., 1995; Yokota et al., 2003). Here we examined the effect of injecting Xnr3 mRNA into one dorsal cell of four-cell-stage FoxH1-depleted embryos. Fig. 7A shows that *Xnr3* mRNA expression partially rescues FoxH1-depleted embryos in causing head formation, but does not rescue correct elongation of the body axis (lower row), while the same dose in control embryos causes convergence extension defects (upper row).

Xnr5 and 6 have been shown by Takahashi et al., to be potent inducers of axial mesoderm and endoderm (Takahashi et al., 2000). Figs 5 and 6 show that *Xnr5* mRNA is expressed at levels at least two-fold higher in FoxH1-depleted embryos compared to control embryos. We asked the question whether this may be a significant increase in expression levels, by injecting a serial dilution of *Xnr5* mRNA into one ventral cell of wild-type four-cell stage embryos. Embryos were frozen at the early gastrula stage and analysed for the level of *Xnr5* mRNA detected by RT-PCR and the induction of the

mesodermal marker *Xbra*. Fig. 7B shows that the lowest dose of *Xnr5* mRNA, 0.6 pg, caused significant induction of *Xbra* mRNA expression, even though the amount of exogenous Xnr5 transcript was barely detectable by RT-PCR at this dilution. Sibling embryos were allowed to develop to tailbud stage, and 0.6 pg Xnr5 caused both head reduction and abnormal

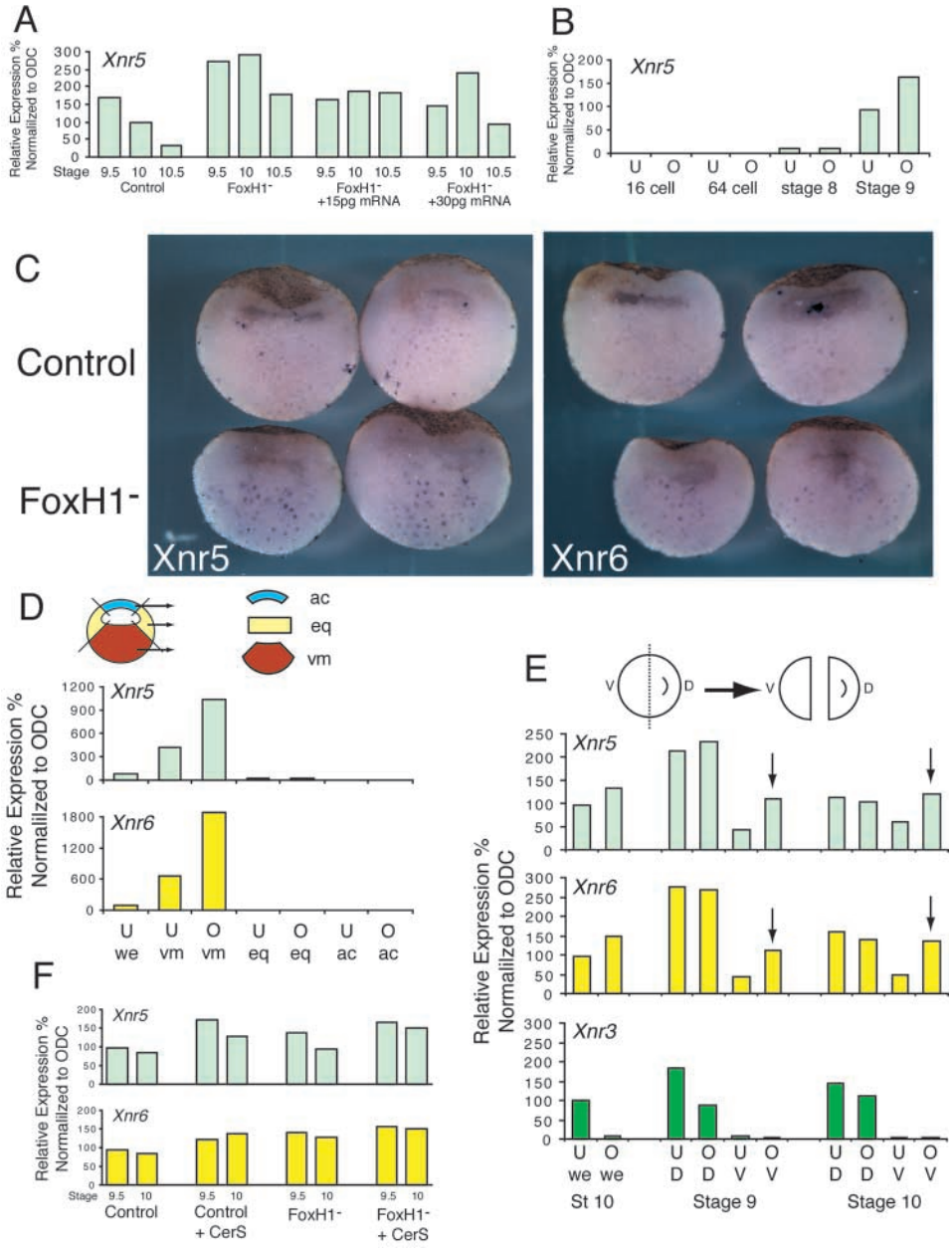


Fig. 5. FoxH1 regulates the correct spatio-temporal expression of *Xnr5* and *6*. (A) The relative expression level of *Xnr5* in control versus FoxH1-depleted embryos at the stages shown assayed by real-time RT-PCR and normalized to ODC. The upregulation of *Xnr5* in FoxH1-depleted embryos is rescued by the injection of 15 or 30 pg *Foxh1* mRNA before maturation. (B) *Xnr5* begins to be upregulated in FoxH1-depleted embryos (O) compared to controls (U) at the late blastula stage (stage 9). (C) In situ hybridization of hemisected control (top row) and FoxH1-depleted late blastulae (bottom row) for *Xnr5* and *Xnr6*, showing the nuclear location and higher levels of expression caused by FoxH1 depletion. (D) The relative expression of *Xnr5* and *6* in wild-type (U) versus FoxH1-depleted (O) explants at the early gastrula stage. ac: animal cap; eq: equatorial explant; vm: vegetal mass; we: one wild-type embryo. RNA was pooled from ten caps, or four vegetal or equatorial explants in each case. *Xnr5* and *6* are both restricted to vegetal cells and are both more abundantly expressed in FoxH1-depleted explants compared to controls. (E) The relative expression levels of *Xnrs 3*, *5* and *6* in control (U) and FoxH1-depleted (O) embryos dissected into dorsal and ventral halves at the late blastula and early gastrula stages. Four ventral or dorsal half embryos were pooled for each RNA sample. U, uninjected; O, antisense oligo injected; D, dorsal halves; V, ventral halves. At both stages, *Xnr5* and *6* are expressed at a higher level in ventral halves of FoxH1-depleted embryos than in control ventral halves. The accuracy of the dissection is shown by the expression of *Xnr3* restricted to the dorsal halves. (F) Nodal signaling was inhibited in control and FoxH1-depleted oocytes by the injection of 500 pg of *CerS* mRNA into oocytes. Embryos were frozen at the late blastula and early gastrula stages and assayed for *Xnr5* and *Xnr6* mRNA expression. *Xnr5* and *6* mRNA are expressed at higher levels in control embryos in which nodal signaling is blocked by CerS.

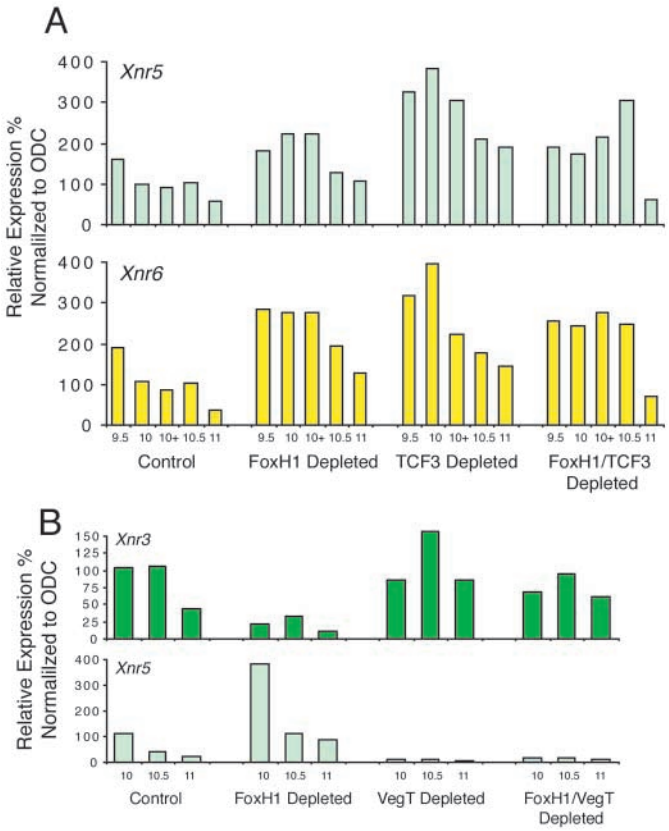


Fig. 6. Maternal FoxH1, XTcf3 and VegT regulate Xnr5 and 6. (A) The relative expression level of *Xnr5* and *6* in wild-type, FoxH1-depleted, XTcf3-depleted and FoxH1/XTcf3-depleted embryos at the stages shown assayed by real-time RT-PCR and normalized to ODC. Both FoxH1 and XTcf3 depletion enhances the expression of *Xnr5* and *6* compared to controls, but the double depletion does not have an additive effect. (B) The relative expression level of *Xnr3* and *5* in wild-type, FoxH1-depleted, VegT-depleted and FoxH1/VegT-depleted embryos at the stages shown assayed by real-time RT-PCR and normalized to ODC. *Xnr5* over-expression in FoxH1-depleted embryos is dependent on maternal VegT. In comparison, *Xnr3* expression is not dependent on VegT.

protrusions. We conclude that a doubling of the expression level of *Xnr5* mRNA in FoxH1-depleted embryos is a significant increase, and is likely to cause developmental abnormalities.

Discussion

Although there have been several studies on the role of FoxH1 in *Xenopus* (Chen et al., 1996; Howell et al., 2002; Watanabe and Whitman, 1999) and other vertebrate embryos (Hoodless et al., 2001; Kunwar et al., 2003; Pogoda et al., 2000; Sirotkin et al., 2000; Trinh et al., 2003; Yamamoto et al., 2001), none have specifically targeted maternal mRNA in the oocyte. To do this we used a phosphorothioate oligonucleotide which depletes the target mRNA in the oocyte and is itself degraded before fertilization. For most genes we have studied in this way, *β-catenin* (Heasman et al., 1994); *VegT* (Zhang et al., 1998); *Creb* (Sundaram et al., 2003); *XTcf3* (Houston et al., 2002); and *plakoglobin* (Kofron et al., 1997), zygotic transcripts begin to accumulate after MBT. This is not the case for *Foxh1*. The embryo inherits a pool of maternal mRNA that is not detectably added to or replaced by zygotic transcripts. Although, in the zebrafish embryo, maternal FoxH1 was not required for normal embryonic development (Pogoda et al., 2000; Sirotkin et al., 2000), we found that the *Xenopus* maternal pool is necessary for head and axis formation.

In these experiments, the degree of depletion of *Foxh1* mRNA by the antisense oligo was to 10-20% of control levels. Since the available antibody is not sensitive enough to detect endogenous FoxH1 protein (data not shown), the extent to which the protein was reduced could not be measured directly. Instead, protein activity was measured by the reduction in the ARE-luciferase activity in FoxH1-depleted embryos compared to controls. Considering that some residual mRNA remained, the extent of reduction of ARE-luciferase activity was surprising. One explanation of the reduction of ARE-luciferase activity could be that maternal protein is only translated in the oocyte, and that the available protein is broken down during the 48-72-hour culture period before fertilization. This explanation is supported by the fact that a shorter incubation time causes a less severe phenotype (data not shown).

Three novel observations came from this study: that *Xnr3* expression is dependent on FoxH1 in a phospho-Smad2-independent fashion, that when ARF activity is severely reduced, the expression of mesodermal and endodermal genes including ‘nodal response genes’ continues in animal caps over-expressing Xnr1 or stimulated by activin, and that *Xnr5* and *6* are negatively regulated by FoxH1.

Xnr3 regulation

Xnr3 was first characterized as an axis-rescuing activity in a *Xenopus* functional screen (Smith et al., 1995), and is expressed in a very restricted spatiotemporal pattern in the organizer region. Loss of function experiments show that it is essential for normal head formation, the convergent extension movements of gastrulation, and for the correct expression of several genes including *Xbra* (Yokota et al., 2003). *Xnr3* expression has been shown to be regulated by the maternal β-catenin/XTcf3 pathway, since interfering with this pathway by expressing dominant negative gsk-3 (Dominguez et al., 1995; He et al., 1995; Pierce and Kimelman, 1995) or by depleting β-catenin (Xanthos et al., 2002) blocks *Xnr3* expression. The experiments presented here suggest that, in wild-type embryos, the de-repression of XTcf3 by β-catenin on the dorsal side is accompanied by transcriptional activation by FoxH1. Transcriptional activation of *Xnr3* by FoxH1 on the ventral side is prevented by XTcf3. In zebrafish, another organizer gene, *goosecoid* is dependent on FoxH1 for its expression (Pogoda et al., 2000). In *Xenopus*, *goosecoid* expression is regulated primarily by VegT and XTcf3/β catenin (Houston et al., 2002) (Fig. 3A).

We do not demonstrate whether the effects of FoxH1 on *Xnr3* are direct or indirect. However, two potential FoxH1 consensus binding sites are present in the published 257-base fragment of the published promoter sequence for *Xnr3* (at positions –238 to –245; 7/8 match and –173 to –180; 8/8 match; data not shown) (McKendry et al., 1997), and *Xnr3* expression occurs immediately after MBT (Xanthos et al., 2001), making it likely that maternal FoxH1 protein regulates *Xnr3* directly.

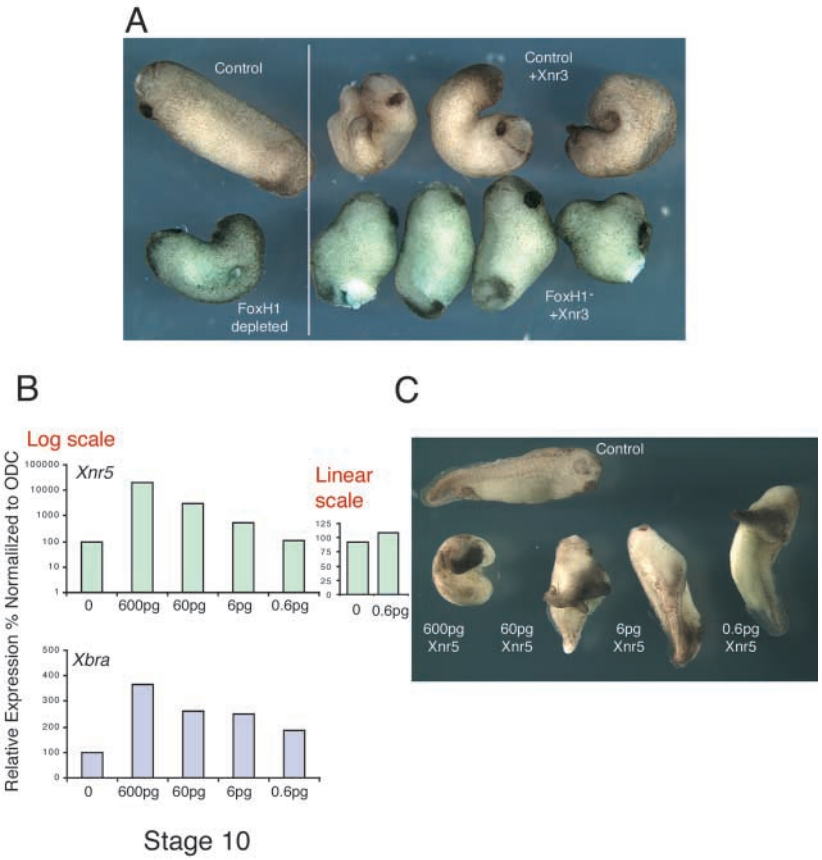


Fig. 7. The misregulation of *Xnr3* and *Xnr5* in FoxH1-depleted embryos contributes to the later abnormalities. (A) Control and FoxH1-depleted embryos injected with 150 pg *Xnr3* mRNA in one dorsal animal cell at the eight-cell stage. Xnr3 overexpression causes convergence extension defects in control embryos and partially rescues head formation in FoxH1-depleted embryos. (B) The relative expression levels of *Xnr5* and *Xbra* mRNA in wild-type early gastrulae injected with 0, 600, 60, 6 and 0.6 pg of *Xnr5* mRNA into one ventral cell at the four-cell stage. (C) The phenotype of sibling embryos of those frozen in (B).

Two lines of evidence suggest a novel aspect of FoxH1 activity; that the transcriptional activation of *Xnr3* by FoxH1 does not require the interaction of FoxH1 with phospho-Smad2. Firstly, *Xnr3* mRNA continues to be expressed in VegT-depleted embryos (Xanthos et al., 2001) and (Fig. 6B), in which we have shown there is no detectable phosphorylation of Smad2 protein (Lee et al., 2001). Secondly, we show here that inactivation of nodal signaling by the expression of the mutant, nodal binding fragment of Cerberus, CerS (Agius et al., 2000) also blocks Smad2 phosphorylation, but does not prevent *Xnr3* expression in wild-type embryos, nor alter its inhibition by the depletion of FoxH1. We have shown (Rex et al., 2002) that expression in whole embryos of a truncated form of the activin receptor, which has dominant negative activity against a range of nodal-like signals, also has no inhibitory effect upon *Xnr3* expression. Previous studies have focused on the activity of FoxH1 in a complex with phospho-Smad2 and 4, and although its potential as a Smad-independent regulator was suggested by the observation that it binds DNA in the absence of Smads (Howell et al., 2002; Yeo et al., 1999), this is, to our knowledge, the first evidence for such a role. Further analysis of the *Xnr3* promoter is required to understand the complexity of its regulation by FoxH1, XTcf3 and β-catenin.

FoxH1 and nodal target gene expression

Previous loss of function studies on *Xenopus* FoxH1, using blocking antibody or a *Drosophila Engrailed–Foxh1*-DNA-binding domain fusion construct, suggested a major role for FoxH1 in regulating the nodal target genes, including *Xlim1*, *Xbra*, *cerberus*, *Mix.2* and *goosecoid* (Watanabe and Whitman, 1999). Here we show that, although the activity of a reporter that consists of a triplet repeat of 50 bp of *Mix.2* promoter containing the FoxH1 binding site is much reduced by FoxH1 depletion, the responses to nodal-type signaling are not correspondingly affected. Two pieces of evidence show this. The expression of nodal target genes in response to *Xnr1* mRNA injection or activin protein induction is unaffected by FoxH1 depletion in animal caps, suggesting that FoxH1 and ARF are not required for this activity. As a second test of the importance of FoxH1, we examined the endogenous expression of nodal response genes including *Xnr1*, *Mix.2*, *goosecoid* and *Xlim1* in FoxH1-depleted embryos, siblings of which developed with a headless phenotype. The level of expression of these genes was reduced, suggesting that FoxH1 modulates their expression levels, but none showed the extreme sensitivity of *Xnr3*. FoxH1 genetic mutants in zebrafish have been shown to affect only a subset of nodal target genes, and to cause reduction rather than complete inhibition of expression (Pogoda et al., 2000; Sirotkin et al., 2000). Our studies support the evidence of Pogoda et al., in zebrafish, and Germain et al., in *Xenopus* (Germain et al., 2000), suggesting that, although FoxH1/Smad2/4 is an important complex in modulating nodal target genes, other transcription factors are also involved. A likely second pathway involves the TAK1-NLK-STAT1 cascade (Ohkawara et al., 2004). The more extreme effects observed using Engrailed repressor constructs and blocking antibody may have been caused by interference with a broader spectrum of genes containing fork-head

domains or, in the case of the antibody, Smad-interacting domains.

In *Xenopus*, the second Fox gene, *Fast3* has been suggested to act, like *Foxh1*, as a mediator of nodal signals. Fast3 has been shown to bind to the same consensus sequence as FoxH1 (Howell et al., 2002). Here, we show that *Fast3* is expressed normally in FoxH1-depleted embryos. Since these embryos lack heads and also lack the ability to activate ARE-luciferase robustly, this suggests that Fast3 does not activate ARE-luciferase or play a role in head formation, although it may regulate the partial expression of nodal-target genes seen here in FoxH1-depleted embryos. It is likely that the expression of each of the 'nodal target genes' is in fact complexly regulated by several transcription factors and co-activators, and repressors, as has been shown recently for the *cerberus* gene (Yamamoto et al., 2003).

Xnr5 regulation

Forkhead genes have generally been shown to be transcriptional activators, but in some contexts may also act as transcriptional repressors (reviewed in Carlsson and Mahlapuu, 2002). Here, depletion of FoxH1 causes an increase in the expression of the two nodals Xnr5 and 6. Xnr5 and 6 were first described as novel Xnrs expressed very early in development before the other family members, in dorsal vegetal cells (Takahashi et al., 2000; Yang et al., 2002). Expression of Xnr5 has been shown to be unaffected by cycloheximide treatment, suggesting that its expression is independent of new protein synthesis (Rex et al., 2002; Takahashi et al., 2000). Previous studies determined that the transcription factors XTcf3 and VegT regulate *Xnr5* expression, and binding sites for these factors were identified in the *Xnr5* promoter. The activity of this promoter has been shown to depend on derepression of XTcf3 by β catenin together with VegT activation (Hilton et al., 2003). Here we show that *Xnr5* mRNA is also prevented from ectopic expression by FoxH1, since FoxH1-depleted embryos express *Xnr5* mRNA in ventral vegetal cells. The effect of FoxH1 on *Xnr5* expression could be direct or indirect. Four potential FoxH1 binding sites were identified in the 785 bp fragment upstream of the TCF binding site in the *Xnr5* promoter (Hilton et al., 2003). A fifth potential site lies in the first intron and this sequence is conserved in both the *Xenopus laevis* and *Xenopus tropicalis* genomic sequence. We show here that while depleting FoxH1 or XTcf3 enhances *Xnr5* expression, the effects of depleting both XTcf3 and FoxH1 are not additive. This may suggest that the two transcription factors interact to regulate Xnr5, and both are essential to form one repressive complex. We confirm here that VegT is essential for the activation of *Xnr5* expression in FoxH1-depleted embryos, just as it is in XTcf3-depleted embryos. We further suggest that this repressive activity of FoxH1 is nodal signalling-dependent. It has been shown that the pattern of Xnr5 expression in the deep endoderm is dynamic at stages 8.5 and 9 (Takahashi et al., 2000). It seems likely, but as yet remains unconfirmed, that this dynamic pattern of *Xnr5* expression relates directly to the dynamic changes in Smad2 phosphorylation known to be taking place at this time (Lee et al., 2001). Further work is required to determine whether FoxH1 binds directly to either of the putative FoxH1-binding sites in Xnr5 and, if so, to show how it acts in an inhibitory fashion when bound.

These results add to our understanding of the maternal regulation of early zygotic gene expression in *Xenopus*. While FoxH1 has been considered as an activator of nodal target gene expression, we show here that nodal responsiveness is not lost in FoxH1-depleted embryos. We find that FoxH1 has specific, non-redundant roles, acting as a co-activator of *Xnr3* together with XTcf3-β catenin, and as a repressor of *Xnr5*. We propose that FoxH1 participates in patterning the mesendoderm by simultaneously repressing *Xnr5* in the ventral region and activating *Xnr3* in the dorsal region. We suggest that these roles of FoxH1 depend on its participating with different transcription factors and co-factors to form different regulatory modules controlling *Xnr5* and *Xnr3* expression. The challenge is to define, for each of the mes-endodermal genes transcribed after MBT, the different combination of maternal and early zygotic transcription factors and co-regulators forming these modules.

This work was supported by NIH RO1 HD 038272 to J. H. Excellent technical support was provided by Kyle Schaible and Stephanie Lang. Thanks to Dr M. Asashima for providing reagents for in situ hybridization and for helpful advice in the in situ protocol.

References

Agius, E., Oelgeschlager, M., Wessely, O., Kemp, C. and De Robertis, E. M. (2000). Endodermal nodal-related signals and mesoderm induction in Xenopus. *Development* **127**, 1173-1183.

Carlsson, P. and Mahlapuu, M. (2002). Forkhead transcription factors: key players in development and metabolism. *Dev. Biol.* **250**, 1-23.

Chen, X., Rubock, M. J. and Whitman, M. (1996). A transcriptional partner for MAD proteins in TGF-beta signalling. *Nature* **383**, 691-696.

Dominguez, I., Itoh, K. and Sokol, S. Y. (1995). Role of glycogen synthase kinase 3 beta as a negative regulator of dorsoventral axis formation in *Xenopus* embryos. *Proc. Natl. Acad. Sci. USA* **92**, 8498-8502.

Germain, S., Howell, M., Esslemont, G. M. and Hill, C. S. (2000). Homeodomain and and winged helix transcription factors recruit activated Smads to distinct promoter elements via a common Smad interaction motif. *Genes Dev.* **14**, 435-451.

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

He, X., Saint-Jeannet, J. P., Woodgett, J. R., Varmus, H. E. and Dawid, I. B. (1995). Glycogen synthase kinase-3 and dorsoventral patterning in *Xenopus* embryos. *Nature* **374**, 617-622.

Heasman, J., Crawford, A., Goldstone, K., Garner-Hamrick, P., Gumbiner, B., McCrea, P., Kintner, C., Noro, C. Y. and Wylie, C. (1994). Overexpression of cadherins and underexpression of beta-catenin inhibit dorsal mesoderm induction in early Xenopus embryos. *Cell* **79**, 791-803.

Hilton, E., Rex, M. and Old, R. (2003). VegT activation of the early zygotic gene Xnr5 requires lifting of Tcf-mediated repression in the *Xenopus* blastula. *Mech. Dev.* **120**, 1127-1138.

Hoodless, P. A., Pye, M., Chazaud, C., Labbe, E., Attisano, L., Rossant, J. and Wrana, J. L. (2001). FoxH1 (Fast) functions to specify the anterior primitive streak in the mouse. *Genes Dev.* **15**, 1257-1271.

Houston, D. W., Kofron, M., Resnik, E., Langland, R., Destree, O., Wylie, C. and Heasman, J. (2002). Repression of organizer genes in dorsal and ventral *Xenopus* cells mediated by maternal XTcf3. *Development* **129**, 4015-4025.

Howell, M., Inman, G. J. and Hill, C. S. (2002). A novel Xenopus Smad-interacting forkhead transcription factor (XFast-3) cooperates with XFast-1 in regulating gastrulation movements. *Development* **129**, 2823-2834.

Huang, H. C., Murtaugh, L. C., Vize, P. D. and Whitman, M. (1995). Identification of a potential regulator of early transcriptional responses to mesoderm inducers in frog embryos. *EMBO J.* **14**, 5965-5973.

Kofron, M., Demel, T., Xanthos, J., Lohr, J., Sun, B., Sive, H., Osada, S., Wright, C., Wylie, C. and Heasman, J. (1999). Mesoderm induction in *Xenopus* is a zygotic event regulated by maternal VegT via TGFbeta growth factors. *Development* **126**, 5759-5770.

Kofron, M., Klein, P., Zhang, F., Houston, D. W., Schaible, K., Wylie, C. and Heasman, J. (2001). The role of maternal axin in patterning the Xenopus embryo. *Dev. Biol.* **237**, 183-201.

Kofron, M., Spagnuolo, A., Klymkowsky, M., Wylie, C. and Heasman, J. (1997). The roles of maternal alpha-catenin and plakoglobin in the early Xenopus embryo. *Development* **124**, 1553-1560.

Kunwar, P. S., Zimmerman, S., Bennett, J. T., Chen, Y., Whitman, M. and Schier, A. F. (2003). Mixer/Bon and FoxH1/Sur have overlapping and divergent roles in nodal signaling and mesendoderm induction. *Development* **130**, 5589-5599.

Lee, M. A., Heasman, J. and Whitman, M. (2001). Timing of endogenous activin-like signals and regional specification of the Xenopus embryo. *Development* **128**, 2939-2952.

McKendry, R., Hsu, S. C., Harland, R. M. and Grosschedl, R. (1997). LEF-1/TCF proteins mediate wnt-inducible transcription from the *Xenopus* nodal-related 3 promoter. *Dev. Biol.* **192**, 420-431.

Ohkawara, B., Shirakabe, K., Hyodo-Miura, J., Matsuo, R., Ueno, N., Matsumoto, K. and Shibuya, H. (2004). The role of the TAK1-NLK-STAT3 pathway in TGF-beta-mediated mesoderm induction. *Genes Dev.* **18**, 381-386.

Osada, S. I., Saijoh, Y., Frisch, A., Yeo, C. Y., Adachi, H., Watanabe, M., Whitman, M., Hamada, H. and Wright, C. V. (2000). Activin/nodal responsiveness and asymmetric expression of a Xenopus nodal-related gene converge on a Fast-regulated module in intron 1. *Development* **127**, 2503-2514.

Pierce, S. B. and Kimelman, D. (1995). Regulation of Spemann organizer formation by the intracellular kinase Xgsk-3. *Development* **121**, 755-765.

Pogoda, H. M., Solnica-Krezel, L., Driever, W. and Meyer, D. (2000). The zebrafish forkhead transcription factor FoxH1/Fast1 is a modulator of nodal signaling required for organizer formation. *Curr. Biol.* **10**, 1041-1049.

Rex, M., Hilton, E. and Old, R. (2002). Multiple interactions between maternally-activated signalling pathways control Xenopus nodal-related genes. *Int. J. Dev. Biol.* **46**, 217-226.

Schier, A. F. (2003). Nodal signaling in vertebrate development. *Annu. Rev. Cell Dev. Biol.* **19**, 589-621.

Sirotkin, H. I., Gates, M. A., Kelly, P. D., Schier, A. F. and Talbot, W. S. (2000). Fast1 is required for the development of dorsal axial structures in zebrafish. *Curr. Biol.* **10**, 1051-1054.

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.

Sundaram, N., Tao, Q., Wylie, C. and Heasman, J. (2003). The role of maternal CREB in early embryogenesis of Xenopus laevis. *Dev. Biol.* **261**, 337-352.

Takahashi, S., Yokota, C., Takano, K., Tanegashima, K., Onuma, Y., Goto, J. and Asashima, M. (2000). Two novel nodal-related genes initiate early inductive events in *Xenopus Nieuwkoop* center. *Development* **127**, 5319-5329.

Trinh, L. A., Meyer, D. and Stainier, D. Y. (2003). The Mix family homeodomain gene bonnie and clyde functions with other components of the nodal signaling pathway to regulate neural patterning in zebrafish. *Development* **130**, 4989-4998.

Watanabe, M. and Whitman, M. (1999). FAST-1 is a key maternal effector of mesoderm inducers in the early Xenopus embryo. *Development* **126**, 5621-5634.

Whitman, M. (2001). Nodal signaling in early vertebrate embryos: themes and variations. *Dev. Cell* **1**, 605-617.

Xanthos, J. B., Kofron, M., Wylie, C. and Heasman, J. (2001). Maternal VegT is the initiator of a molecular network specifying endoderm in Xenopus laevis. *Development* **128**, 167-180.

Xanthos, J. B., Kofron, M., Tao, Q., Schaible, K., Wylie, C. and Heasman, J. (2002). The roles of three signaling pathways in the formation and function of the Spemann Organizer. *Development* **129**, 4027-4043.

Yamamoto, M., Meno, C., Sakai, Y., Shiratori, H., Mochida, K., Ikawa, Y., Saijoh, Y. and Hamada, H. (2001). The transcription factor FoxH1 (FAST) mediates Nodal signaling during anterior-posterior patterning and node formation in the mouse. *Genes Dev.* **15**, 1242-1256.

Yamamoto, S., Hikasa, H., Ono, H. and Taira, M. (2003). Molecular link in the sequential induction of the Spemann organizer: direct activation of the cerberus gene by Xlim-1, Xotx2, Mix.1, and Siamois, immediately downstream from Nodal and Wnt signaling. *Dev. Biol.* **257**, 190-204.

Yang J., Tan C., Darken R. S., Wilson, P. A. and Klein, P. S. (2002). β catenin/Tcf-regulated transcription prior to the mid-blastula transition. *Development* **129**, 5743-5752.

Yeo, C. Y., Chen, X. and Whitman, M. (1999). The role of FAST-1 and Smads in transcriptional regulation by activin during early Xenopus embryogenesis. *J. Biol. Chem.* **274**, 26584-26590.

Yokota, C., Kofron, M., Zuck, M., Houston, D. W., Isaacs, H., Asashima, M., Wylie, C. C. and Heasman, J. (2003). A novel role for a nodal-related protein; Xnr3 regulates convergent extension movements via the FGF receptor. *Development* **130**, 2199-2212.

Zhang, J., Houston, D. W., King, M. L., Payne, C., Wylie, C. and Heasman, J. (1998). The role of maternal VegT in establishing the primary germ layers in Xenopus embryos. *Cell* **94**, 515-524.

Zuck, M. V., Wylie, C. C. and Heasman, J. (1998). Maternal mRNAs in *Xenopus* embryos: an antisense approach. In *A Comparative Methods Approach to the Study of Oocytes and Embryos* (ed. J. D. Richter), pp. 341-354. Oxford: Oxford University Press.