New roles for FoxH1 in patterning the early embryo

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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 XTcf3β 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 (Harland, 1991) using BM Purple as substrate (Roche). The RNase AT1 digestion was omitted from the protocol (Harland, 1991) using BM Purple as substrate (Roche). The RNase AT1 digestion was omitted from the protocol (Harland, 1991) using BM Purple as substrate (Roche).

Oligos and mRNAs
The antisense FoxH1 oligo used was an 18-mer 5′-C*A*G*G*CTTCTACGATC*G*A*G*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*G*GACATGTCCT*T*G*G*G*C-3′) and XTcf3 (5′-C*G*A*G*G*GATCCCGACT*T*G*G*G*C-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 NolI, 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 Mx2 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 un.injected 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
neural 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 and begins to be expressed after the mid-blastula transition mRNA is not supplemented or replaced by zygotic transcripts.

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

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

<table>
<thead>
<tr>
<th>PCR primer pair</th>
<th>Origin</th>
<th>Sequence</th>
<th>Denaturing temperature (°C)</th>
<th>Annealing temperature (°C)/time (sec)</th>
<th>Extension temperature (°C)/time (sec)</th>
<th>Acquisition temperature (°C)/time (sec)</th>
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<td>Chordin</td>
<td>XMMR*</td>
<td>U: 5′-AACGTGCCAGAAGCTGATGGT-3′ D: 5′-GGCAAGATTCTGAGGCTTGAC-3′</td>
<td>95</td>
<td>55/5</td>
<td>72/12</td>
<td>81/3</td>
</tr>
<tr>
<td>Cerberus</td>
<td>Heasman et al., 2000</td>
<td>U: 5′-GCTTGGAAACTTCTGCTT-3′ D: 5′-CTGATGGAAGGATGACATT-3′</td>
<td>95</td>
<td>60/5</td>
<td>72/20</td>
<td>81/3</td>
</tr>
<tr>
<td>dikkopa</td>
<td>New</td>
<td>U: 5′-CAAGAGCAGGACAGAGA-3′ D: 5′-TCAGGGGAGACGAGCC-3′</td>
<td>95</td>
<td>56/5</td>
<td>72/10</td>
<td>82/3</td>
</tr>
<tr>
<td>Fast3</td>
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<td>U: 5′-AACCCCCACAGTGAGGAAGA-3′ D: 5′-CTCATTTGCGCATGACA-3′</td>
<td>95</td>
<td>56/5</td>
<td>72/14</td>
<td>88/3</td>
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<tr>
<td>Fgf8</td>
<td>Koifon et al., 1999</td>
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<td>95</td>
<td>55/5</td>
<td>72/14</td>
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<td>95</td>
<td>56/5</td>
<td>72/12</td>
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<td>New</td>
<td>U: 5′-TCACCAGCAAACAAGTGG-3′ D: 5′-TTCCACTTTGCGATCTTTC-3′</td>
<td>95</td>
<td>55/5</td>
<td>72/11</td>
<td>82/3</td>
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<td>XMMR*</td>
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<td>60/5</td>
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<td>55/5</td>
<td>72/10</td>
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<td>Mixer</td>
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<td>55/5</td>
<td>72/12</td>
<td>83/3</td>
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<td>Heasman et al., 2000</td>
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<td>95</td>
<td>55/5</td>
<td>72/16</td>
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<td>Xbra</td>
<td>Sun et al., 1999</td>
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<td>72/8</td>
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<td>55/5</td>
<td>72/11</td>
<td>85/3</td>
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<td>55/5</td>
<td>72/10</td>
<td>76/3</td>
</tr>
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<td>95</td>
<td>55/5</td>
<td>72/10</td>
<td>77/3</td>
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<td>Xnr3 v.21</td>
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<td>95</td>
<td>56/5</td>
<td>72/9</td>
<td>79/3</td>
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<td>55/5</td>
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<td>New</td>
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<td>62/5</td>
<td>72/20</td>
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<td>Sox217α</td>
<td>Xanthos et al., 2001</td>
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<td>95</td>
<td>58/5</td>
<td>72/8</td>
<td>85/3</td>
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*XMMR, Xenopus Molecular Marker Resource (http://www.xenbase.org/xmmr/Marker_pages/primers.html).  
1v.2, new version of primer designed with primer3 web based program (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3 www.cgi).
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 (Sirokin 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
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
Maternal Foxh1 in Xenopus development

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type and maternal FoxH1-depleted embryos, for other genes that were clearly regulated by FoxH1 (Fig. 4E). All the endodermal and meso-endodermal 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 30 pg 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.

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 βcatenin 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 Xnr3 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 development.

**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 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. (E) The relative expression levels of Xnr3, 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.
and Creb (Sundaram et al., 2003); -catenin before fertilization. For most genes we have studied in this way, depletes the target mRNA in the oocyte and is itself degraded.

This we used a phosphorothioate oligonucleotide which, 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 of these genes including Xbra (Yokota et al., 2003). Xnr3 expression has been shown to be regulated by the maternal /catenin/XTcf3 pathway, since interfering with this pathway by depleting -catenin (Xanthos et al., 2002) blocks Xnr3 expression. The experiments presented here suggest that, in wild-type embryos, the de-repression of XTcf3 by /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/ /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.

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 of these genes including 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 Xnr3 and 6 are negatively regulated by FoxH1.
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 (Okawara 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 Xnr5s 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 depletion of 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.

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**References**


