Published in final edited form as:
Development. 2006 December ; 133(24): 4827–4838.

FoxD3 Regulation of Nodal in the Spemann Organizer is Essential for *Xenopus* Dorsal Mesoderm Development

Aaron B. Steiner, **Mark J. Engleka**, **Qun Lu**, **Eileen C. Piwarzyk**, **Sergey Yaklichkin**, **Julie L. Lefebvre**, **James W. Walters**, **Liliam Pineda-Salgado**, **Patricia A. Labosky**[†], and **Daniel S. Kessler**[*]

Department of Cell and Developmental Biology University of Pennsylvania School of Medicine 1110 BRB 2/3, 421 Curie Boulevard Philadelphia, PA 19104, USA Tel: 215-898-1478 Fax: 215-573-7601 Email: kesslerd@mail.med.upenn.edu

SUMMARY

Induction and patterning of the mesodermal germ layer is a key early step of vertebrate embryogenesis. We report that *FoxD3* function in the *Xenopus* gastrula is essential for dorsal mesodermal development and for *Nodal* expression in the Spemann organizer. In embryos and explants, FoxD3 induced mesodermal genes, convergent extension movements, and differentiation of axial tissues. Engrailed-FoxD3, but not VP16-FoxD3, was identical to native FoxD3 in mesoderm-inducing activity, indicating that FoxD3 functions as a transcriptional repressor to induce mesoderm. Antagonism of FoxD3 with VP16-FoxD3 or morpholino-knockdown of FoxD3 protein resulted in a complete block to axis formation, a loss of mesodermal gene expression, and an absence of axial mesoderm, indicating that transcriptional repression by FoxD3 is required for mesodermal development. FoxD3 induced mesoderm in a non-cell-autonomous manner, indicating a role for secreted inducing factors in the response to FoxD3. Consistent with this mechanism, FoxD3 was necessary and sufficient for the expression of multiple *Nodal*-related genes, and inhibitors of Nodal signaling blocked mesoderm induction by FoxD3. Therefore, FoxD3 is required for *Nodal* expression in the Spemann organizer and this function is essential for dorsal mesoderm formation.

Keywords

Xenopus; FoxD3; Forkhead; Nodal; mesoderm; transcription

INTRODUCTION

Formation of the vertebrate body plan is a process of self-organization, with the fertilized egg undergoing subdivision and induction to set up the primary germ layers and organizing centers, leading to morphogenesis, differentiation and axis formation. While localized maternal factors initiate regional gene expression and bias cell fate, zygotic transcriptional programs are required to determine cell fate and confer stable embryonic pattern. During gastrulation, these transcriptional networks undergo positive and negative feedback, reinforcing lineage-specific gene expression and refining boundaries between developmental compartments. In this way developmental programs are selected and maintained in the gastrula, providing a stable spatial framework for further elaboration of the body plan (reviewed in Harland and Gerhart, 1997; Heasman, 2006; De Robertis et al., 2000). For example, in *Xenopus* mesoderm formation, Nodal signals are subjected to multiple positive and negative inputs that reinforce pathway

[*]Author for correspondence (e-mail: kesslerd@mail.med.upenn.edu)
[†]Present address: Center for Stem Cell Biology, Department of Cell and Developmental Biology, Vanderbilt University, Nashville, TN, 37232, USA

activity in the mesodermal domain and exclude pathway activity in the adjacent ectodermal region (Schier and Shen, 2000; Whitman, 2001).

The Fox gene family comprises a large and functionally diverse group of *forkhead*-related transcriptional regulators, many of which are essential for metazoan embryogenesis and physiology (Carlsson and Mahlapuu, 2002; Lehmann et al., 2003; Pohl and Knochel, 2005). FoxD3 is a member of the Fox family that has multiple roles in the vertebrate embryo, including regulation of neural crest development and maintenance of mammalian stem cell lineages. *FoxD3* orthologs in *Xenopus* (Xfd6/Xfkh6), zebrafish (Fkd6), chick (Cwh3) and mouse (Genesis/Hfh2) are expressed in the neural crest (Dirksen and Jamrich, 1995; Scheucher et al., 1995; Lef et al., 1996; Sutton et al., 1996; Freyaldenhoven et al., 1997a; Labosky and Kaestner, 1998; Odenthal and Nusslein-Volhard, 1998; Yamagata and Noda, 1998; Kelsh et al., 2000). Studies in *Xenopus* and chick indicate that FoxD3 regulates the determination, migration, survival and/or differentiation of a number of neural crest lineages (Dottori et al., 2001; Kos et al., 2001; Pohl and Knochel, 2001; Sasai et al., 2001; Cheung et al., 2005; Whitlock et al., 2005; Lister et al., 2006; Stewart et al., 2006). A role in the neural crest is further supported by the association of a human *FOXD3* promoter sequence variant with autosomal dominant vitiligo, a pigmentation disorder caused by defects in the melanoblast lineage (Alkhateeb et al., 2005).

Foxd3 is also expressed in the preimplantation mouse embryo, in mouse and human embryonic stem cells, and in mouse trophoblast stem cells (Sutton et al., 1996; Pera et al., 2000; Hanna et al., 2002; Tompers et al., 2005). *Foxd3* null embryos have a severe reduction of epiblast cell number and die by 6.5 dpc, and *Foxd3* null trophoblast progenitors are defective in both self-renewal and differentiation. In addition, neither embryonic stem cell lines nor trophoblast stem cell lines can be established from *Foxd3* null embryos (Hanna et al., 2002; Tompers et al., 2005). The requirement for *Foxd3* in both embryonic and trophoblast stem cells suggests that *Foxd3* may also be required in multipotent neural crest stem cells, but it is not yet known if the molecular and developmental functions of Foxd3 are similar in these diverse progenitor populations.

Prior to expression in the neural crest, *FoxD3* is expressed in the Spemann organizer, the zebrafish shield, and the chick and mouse node (Supplementary Material Fig. S1) (Labosky and Kaestner, 1998; Odenthal and Nusslein-Volhard, 1998; Yamagata and Noda, 1998; Pohl and Knochel, 2001; Sasai et al., 2001; Yaklichkin et al., 2003), the gastrula signaling center that controls germ layer patterning, morphogenesis and axis formation (reviewed in Harland and Gerhart, 1997; De Robertis et al., 2000). Here we report that FoxD3 function in the Spemann organizer is essential for dorsal mesodermal development. FoxD3 functions as a transcriptional repressor to induce dorsal mesoderm and axis formation, and antagonism or knockdown of FoxD3 results in severe axial defects and loss of dorsal mesodermal gene expression. FoxD3 induction of mesoderm is non-cell autonomous and requires the Nodal signaling pathway. Consistent with the coexpression of *FoxD3* and *Nodal* genes in the organizer, FoxD3 is necessary and sufficient for the expression of several *Nodal*-related genes. Taken together, our results demonstrate a novel mode of Nodal regulation in the Spemann organizer, where transcriptional repression by FoxD3 maintains *Nodal* expression to promote mesoderm induction and axial development.

MATERIALS AND METHODS

Embryos and Microinjection

Embryos were collected, fertilized, injected and cultured as previously described (Yao and Kessler, 1999), and embryonic stage was determined according to Nieuwkoop and Faber (1967) (Nieuwkoop and Faber, 1967). Dorsal and ventral blastomeres were identified by

pigmentation differences (Klein, 1987). Explants were prepared using a Gastromaster microsurgery instrument (Xenotek Engineering). Capped, in vitro transcribed RNA for microinjection was synthesized from linearized template DNA using the Message Machine kit (Ambion) and 10 nl of RNA solution was injected. Templates for in vitro transcription were pCS2-FoxD3, pCS2-mFoxD3, pCS2-Eng-FoxD3, pCS2-VP16-FoxD3, pCS2-FoxD3(N140A/H144A), pCS2-Eng-FoxD3(N140A/H144A), pCS2-VP16-FoxD3(N140A/H144A), pCS2-NLS-FoxD3WH, pCS2-FoxD3-utr (this study), pCS2-Eng, pCS2-VP16, pCS2-MT-SID (Chen et al., 1997), pCS2-Cer-S (Piccolo et al., 1999), pCS2-Xnr1 (Sampath et al., 1997), and pCS2-VegTΔUTR (Engleka et al., 2001).

FoxD3 Expression Constructs

The FoxD3 constructs described in this study were generated by subcloning into pCS2+, pCS2-NLS, or pCS2-GFP (Rupp et al., 1994). A FoxD3 cDNA clone (nucleotides 105-1308) containing the ORF flanked by 67 nucleotides of 5'UTR and 21 nucleotides of 3'UTR was obtained by RT-PCR of tailbud stage mRNA using primers derived from the published sequence of *Xenopus* FoxD3 (Dirksen and Jamrich, 1995). This subclone, referred to in this study as pCS2-FoxD3, pCS2-xFoxD3 or pCS2-FoxD3+utr, was used to generate the additional FoxD3 constructs. A detailed description of the *Xenopus* FoxD3 constructs used in this study is provided in Supplementary Material (Fig. S2). The mouse Foxd3 construct (pCS2-mFoxD3) was generated by subcloning an EcoRI genomic fragment containing the ORF flanked by 75 nucleotides of 5'UTR and 600 nucleotides of 3'UTR (Labosky and Kaestner, 1998).

Morpholino Oligonucleotides

The FoxD3 antisense morpholino oligonucleotide (FoxD3MO) is complementary to nucleotides 158-181 of *Xenopus* FoxD3 (5′-ACAGGGTCATTCCAGTTACGCTCC-3′) and was injected at 10-100 ng per embryo (Gene Tools). As a control, embryos were injected with equal doses of a mismatch morpholino oligonucleotide (misMO) complementary to nucleotides 158-181 of FoxD3 at all but 5 positions (5'-ACAcGGTgATTCaAGTTACcCTgC-3').

In Situ Hybridization, Immunocytochemistry and Histology

For wholemount in situ hybridization, embryos were fixed and hybridized with antisense, digoxygenin-labeled RNA probes as described (Sive et al., 2000). Hybridized probe was detected using alkaline phosphatase-conjugated anti-digoxygenin Fab fragments (Boehringer-Mannheim) and BMpurple (Boehringer-Mannheim) as substrate for color development. Antisense probes were synthesized from linearized plasmid DNA using the Megascript kit (Ambion) supplemented with 2 mM digoxygenin-11-UTP. Templates for in situ probes were pGEM-Xbra (Wilson and Melton, 1994), pCS2-Chd (Sasai et al., 1994), pBS-Dlx3 (Feledy et al., 1999), pGEM-Gsc (Cho et al., 1991), pT7blue-Mixer (Engleka et al., 2001), pBS-Opl (Kuo et al., 1998), pBS-Xnr1, pBS-Xnr2 (Jones et al., 1995), and pGEM-Xwnt8 (Sokol et al., 1991). For serial section immunocytochemistry, embryos were embedded in paraplast as described (Sive et al., 2000) and 15 μm sections were stained with monoclonal antibodies specific for muscle (12/101) (Kintner and Brockes, 1984), notochord (Tor70) (Bolce et al., 1992), or neural tissue (4d) (Watanabe et al., 1986), and HRP-coupled secondary antibody. Positive staining was visualized with VIP, DAB+Ni or DAB as HRP substrates (Vector Laboratories). For histology, 10 μm sections were prepared from paraplast-embedded embryos and explants, and dewaxed sections were stained with hematoxylin/eosin before coverslipping with Permount. For double-staining, samples were processed for in situ hybridization, and following the chromogenic reaction, samples were fixed and processed for immunocytochemistry as described (Sive et al., 2000).

Reverse Transcription-Polymerase Chain Reaction and Western Analysis

For RT-PCR, total RNA was isolated using the RNAqueous kit (Ambion), and cDNA synthesis and PCR were performed as described (Wilson and Melton, 1994). Radiolabelled PCR products were resolved on 5% native polyacrylamide gels. PCR primers and cycle parameters were as described for EF1α, Xbra, Xwnt8, Muscle Actin, NCAM (Wilson and Melton, 1994), Collagen Type II (Agius et al., 2000), MyoD (Rupp et al., 1994), Xnr1, Xnr2 (Sampath et al., 1997), Xnr4 (Joseph and Melton, 1997) and Derriere (Sun et al., 1999). For western analysis, injected embryos were lysed (10 μl per embryo) in 0.1 M Tris-HCl (pH 6.8) supplemented with protease inhibitors. The extracts were cleared by centrifugation and half an embryo equivalent was loaded per well. An affinity-purified anti-*Xenopus* FoxD3 polyclonal antibody (Supplementary Material Fig. S1) (this study; Tompers et al., 2005), was used at a 1:1000 dilution and was detected with a 1:3000 dilution of anti-rabbit IgG-peroxidase by chemiluminescence (Amersham). As a loading control, stripped blots were analyzed with a monoclonal antibody against MAPK (ERK1/2) (Sigma). For analysis of phospho-Smad2, animal explants lysates were prepared as previously described (Lee et al., 2001) and phospho-Smad2 was detected using a phospho-specific monoclonal antibody (Cell Signaling). As a loading control, stripped blots were analyzed with a polyclonal antibody against Smad2/3 (Cell Signaling).

RESULTS

FoxD3 induction of axis formation

The developmental function of *FoxD3* was examined by ectopic expression in ventral mesoderm, outside of the normal *FoxD3* expression domain in the gastrula, and the effect on axis formation was assessed. *FoxD3* RNA was injected into a single ventral blastomere at the 4-cell stage and the embryos were examined morphologically at the tadpole stage. In the dose range of 100-300 pg, a majority of the injected embryos (78%, n=165) displayed abnormal axial development (Fig. 1). At higher doses the predominant phenotype was the presence of anterior axial duplications that included ectopic eyes and head structures (Fig. 1B). At low doses, ectopic posterior structures were observed that had the appearance of accessory tail structures (Fig. 1C). To identify the cell types present in FoxD3-induced ectopic structures, embryos were serially sectioned and adjacent sections were stained with tissue-specific antibodies for somitic muscle (12/101) (Kintner and Brockes, 1984), notochord (Tor70) (Bolce et al., 1992), and neural tissue (4d) (Watanabe et al., 1986). All affected embryos contained a mass of ectopic muscle (Fig. 1D) and an expansion and disorganization of the neural tube (Fig. 1F). Embryos with ectopic anterior structures displayed two, and sometimes three, notochords (Fig. 1E). Consistent with the observed effects on axis formation, expression of FoxD3 in ventral marginal zone explants induced markers of dorsal mesoderm and differentiation of dorsal axial tissues (Supplementary Materials Fig. S3).

The influence of ectopic FoxD3 on mesodermal pattern was also examined at the gastrula stage. At the four-cell stage, *FoxD3* mRNA was injected into a single ventral blastomere and embryos were collected for whole-mount in situ hybridization at the early gastrula stage. Consistent with the axial effects, FoxD3 induced ectopic expression of *Goosecoid*, an organizer marker (Fig. 1H). The results demonstrate that FoxD3 is sufficient for ectopic dorsal mesoderm formation and suggest a role for FoxD3 in endogenous mesoderm formation and/or patterning, consistent with the expression of *FoxD3* in the Spemann organizer. We note that the response to FoxD3 is similar to activation of the Smad2 pathway by TGFß-related proteins, which induce dorsal mesoderm formation, and Wnt activation of the ~catenin pathw ay, which dorsalizes ventral mesoderm (Heasman, 2006).

Dorsal mesoderm induction by FoxD3

To determine whether FoxD3 is sufficient for the induction of mesoderm, FoxD3 was expressed in animal explants that normally differentiate as atypical epidermis. At the one-cell stage, *FoxD3* mRNA was injected into the animal pole and explants isolated at the late blastula stage were cultured to the midgastrula or tailbud stages. In contrast to control explants that remain spherical and form atypical epidermis, explants expressing either *Xenopus* or mouse FoxD3 underwent convergent extension movements and were highly elongated, indicative of dorsal mesoderm induction (Symes and Smith, 1987) (Fig. 2A-C). To confirm that mesoderm induction had occurred, explants were analyzed by immunocytochemistry, histology and RT-PCR. The presence of differentiated somitic muscle was detected at the tailbud stage by whole-mount immunocytochemistry with a muscle-specific monoclonal antibody (12/101) (Kintner and Brockes, 1984). While control explants had no detectable muscle, nearly all FoxD3-expressing explants (90%, n=20) contained abundant muscle (Fig. 2D,E). The explants were subsequently sectioned and counterstained with hematoxylin/eosin for histological analysis. FoxD3-expressing explants contained somitic muscle, notochord, and neural tissue, while control explants contained only ciliated epidermis (Fig. 2G,H). Gene expression was examined by RT-PCR at the midgastrula and tailbud stages. FoxD3 induced the expression of *Brachyury* (pan-mesodermal), *Goosecoid* (dorsal mesoderm/organizer) and *Xwnt8* (ventrolateral mesoderm) at the midgastrula stage. Additional organizer markers, including *Chordin* and *Noggin*, were also induced by FoxD3 (data not shown). At the tailbud stage, FoxD3 induced the expression of *Muscle Actin* (somitic mesoderm), *Lim1* and *Pax8* (pronephros), and *NCAM* (pan-neural), but not markers of heart (*Nkx2.5* and *Tbx5*) or blood (*AML* and α*T4-Globin*) (Fig. 2J and data not shown). Identical results were obtained for the *Xenopus* and mouse orthologs of *FoxD3* (Fig. 2C,J and data not shown). Therefore, FoxD3 is sufficient for mesodermal gene expression and the induction of differentiated axial mesoderm. This mesoderm-inducing activity of FoxD3 is most similar to the Smad2-activating TGFß-related ligands, including Activin, Vg1 and Nodal (Heasman, 2006).

FoxD3 functions as a transcriptional repressor to induce mesoderm

As a member of the Forkhead family of transcriptional regulators, it is predicted that FoxD3 induces mesoderm by transcriptional activation or repression of specific target genes. To determine the transcriptional activity of FoxD3 responsible for mesoderm induction, the activity of chimeric FoxD3 proteins containing the FoxD3 DNA-binding domain fused to defined transcriptional regulatory domains was examined. In this strategy, the specific DNA-binding domain delivers a strong activator or repressor to endogenous target genes and stimulates or inhibits their transcription (Conlon et al., 1996; Kessler, 1997). Chimeric proteins were generated containing the HSV VP16 activator domain (Sadowski et al., 1988; Triezenberg et al., 1988) or the *Drosophila* Engrailed repressor domain (Jaynes and O'Farrell, 1991; Han and Manley, 1993; Badiani et al., 1994) fused to the winged helix DNA-binding domain of FoxD3 (Fig. 3A). The mesoderm-inducing activities of the Engrailed repressor fusion protein (Eng-FoxD3) and the VP16 activator fusion protein (VP16-FoxD3) were examined by expression in animal explants. Like native FoxD3, Eng-FoxD3 induced convergent extension movements, while VP16-FoxD3 did not have this effect (Fig. 3B-E). Consistent with the morphology of the explants, Eng-FoxD3 induced the expression of *Muscle Actin* and *Collagen Type II*, a notochord marker, while VP16-FoxD3 did not activate these axial mesoderm markers (Fig. 3F). Histological analysis at the tailbud stage and RT-PCR analysis at the gastrula stage confirmed that the mesoderm-inducing activities of Eng-FoxD3 and native FoxD3 were indistinguishable (data not shown). Furthermore, like native FoxD3, Eng-FoxD3 induced ectopic dorsal mesoderm when expressed in the ventral marginal zone (data not shown). The results suggest that FoxD3 functions as a transcriptional repressor to induce mesoderm. In a Gal4-UAS transcriptional assay, FoxD3 repressed basal transcription of a luciferase reporter ~15-fold in animal explants at the gastrula stage (data not shown). This result confirms that

FoxD3 functions as a transcriptional repressor, consistent with previous studies of *FoxD3* orthologs in cell culture and in the neural crest lineage (Sutton et al., 1996; Freyaldenhoven et al., 1997b; Pohl and Knochel, 2001; Sasai et al., 2001).

The observation that FoxD3 functions as a repressor to induce mesoderm suggested that VP16-FoxD3 may have the ability to antagonize FoxD3 by activating target genes normally repressed by FoxD3. To assess the potential inhibitory activity of VP16-FoxD3, native FoxD3 and VP16-FoxD3 were coexpressed in animal explants and the induction of mesodermal markers was examined. While FoxD3 induced *Muscle Actin* and *Collagen Type II*, this response was fully inhibited by coexpression of VP16-FoxD3 (Fig. 3G). Therefore, an "activator" form of FoxD3 antagonizes the activity of native FoxD3. As discussed below, this result raises the possibility of using VP16-FoxD3 to inhibit the function of endogenous FoxD3.

For the chimeric proteins, the FoxD3 DNA-binding domain is predicted to deliver the activator or repressor domains to specific target genes normally regulated by FoxD3. To confirm that DNA-binding activity is required for the function of the native and chimeric forms of FoxD3, conserved DNA contact residues were mutated (N140A/H144A) to generate DNA-binding inactive forms of native FoxD3 and the chimeric proteins (Supplemental Material Fig. S2). In animal explants the DNA-binding inactive forms of FoxD3 and Eng-FoxD3 did not induce mesoderm, and the VP16-FoxD3 mutant did not inhibit the activity of native FoxD3 (data not shown). In addition, the individual domains that comprise the chimeric FoxD3 proteins (FoxD3 DNA-binding domain, VP16 activator and Engrailed repressor) had no activity (data not shown). Therefore, sequence-specific DNA-binding activity is required for the function of native and chimeric forms of FoxD3.

Taken together, the results indicate that FoxD3 functions as a transcriptional repressor to induce mesoderm. Beyond defining the transcriptional activity of FoxD3 responsible for mesoderm induction, the results have an unexpected implication for the regulation of mesodermal development. The ability of FoxD3 and Eng-FoxD3 to induce mesoderm argues for the presence of a negative regulator of mesoderm formation that is repressed by FoxD3. This suggests that the establishment of mesoderm in *Xenopus* may involve transcriptional repression of a mesodermal inhibitor.

FoxD3 is required for axial and mesodermal development

Loss-of-function analysis can be accomplished in *Xenopus* by injection of an antisense morpholino oligonucleotide (MO) that specifically blocks translation of a target mRNA (Summerton and Weller, 1997; Heasman et al., 2000). To determine the requirement for *FoxD3* function in *Xenopus* mesodermal development, a MO was designed that is complementary to the *FoxD3* mRNA in the region of the initiator methionine codon (Fig. 4A). FoxD3MO is predicted to form a stable heteroduplex with *FoxD3* mRNA and block translational initiation (Summerton and Weller, 1997). To assess the efficacy of FoxD3MO, embryos were injected with *FoxD3* mRNA and FoxD3MO or a control MO containing five mismatches with the FoxD3 target sequence (mismatch MO), and FoxD3 translation in animal explants was examined by western blot analysis (Fig. 4B). Translation of a *FoxD3* RNA containing the entire target sequence (FoxD3+utr) was blocked by FoxD3MO, while a *FoxD3* RNA lacking the 5'UTR target sequence (FoxD3-utr) was translated normally. The mismatch MO did not inhibit the translation of either *FoxD3* RNA. The ability of FoxD3MO to interfere with the mesoderm-inducing activity of FoxD3 was examined in animal explants. Consistent with the observed translational block, FoxD3MO inhibited the induction of *Muscle Actin* by FoxD3+utr, but did not affect the response to FoxD3-utr (Fig. 4C). Mismatch MO did not block induction by either RNA. To assess the ability of FoxD3MO to inhibit translation of endogenous FoxD3, embryos injected with FoxD3MO or mismatch MO were analyzed by western blotting at the midgastrula stage (Fig. 4D). A single major protein identical in size to

overexpressed *Xenopus* FoxD3 was detected in uninjected and mismatch MO-injected embryos, and FoxD3MO resulted in an ~10-fold reduction in protein levels. This striking inhibition of endogenous FoxD3 translation suggests that FoxD3MO injection results in a complete or near complete loss-of-function for FoxD3.

The developmental requirement for *FoxD3* was examined using VP16-FoxD3 and FoxD3MO. It is predicted that VP16-FoxD3 will antagonize FoxD3 function by activating target genes normally repressed by endogenous FoxD3, and that FoxD3MO will inhibit translation of endogenous FoxD3. At the 4-cell stage, each blastomere was injected in the marginal region with VP16-FoxD3 or FoxD3MO (total dose 1 ng and 60 ng, respectively). Severe axial defects, including loss of head, trunk and tail structures, were observed at the tailbud stage for both VP16-FoxD3 (81%, n=289) and FoxD3MO (74%, n=311) (Fig. 4H,J). Histological analyses showed a great reduction or complete absence of somitic muscle, notochord and neural tube in embryos injected with VP16-FoxD3 or FoxD3MO (Fig. 4I,K), and this was confirmed by immunocytochemistry with antibodies specific for each axial tissue (data not shown). At lower doses of VP16-FoxD3 and FoxD3MO (0.3 ng and 20 ng, respectively), head structures did not form, but trunk and tail development was normal, and at the highest doses (2 ng and 100 ng, respectively) embryos initiated gastrulation, but did not complete blastopore closure (data not shown). As controls, the mismatch MO and a DNA-binding inactive form of VP16-FoxD3 (N140A and H144A) resulted in a slight anterior reduction in a few embryos (5%, n=88 and 6%, n=120, respectively), but more severe effects were not observed (Fig. 4L,M and data not shown). Therefore, axis formation is disrupted by two distinct methods for FoxD3 inhibition, suggesting that axial development is dependent on FoxD3 function.

The inhibition of axis formation by VP16-FoxD3 and FoxD3MO is predicted to result from a specific block of endogenous FoxD3 function. To determine the specificity of FoxD3 inhibition, FoxD3 was coinjected with VP16-FoxD3 or FoxD3MO in an attempt to rescue axis formation (Table 1 and Supplementary Material Fig. S4). While the majority of VP16-FoxD3-injected embryos had severe axial defects (73%, n=44), only a minority displayed defects with FoxD3 coinjection (13%, n=61). Similarly, the axial defects caused by FoxD3MO (79%, n=38) were rescued by *FoxD3* RNA lacking the antisense target sequence (FoxD3-utr) (9%, n=54), but not by *FoxD3* RNA containing the target sequence (FoxD3+utr) (67%, n=49). As controls, injection of both dorsal blastomeres with *FoxD3* RNA or mismatch MO did not perturb axis formation. The rescue of axis formation by FoxD3 indicates that VP16-FoxD3 and FoxD3MO are specific inhibitors of endogenous FoxD3.

To determine the developmental origin of the axial defects caused by VP16-FoxD3 and FoxD3MO injection, gene expression patterns were examined at the gastrula stage. At the 4-cell stage, each blastomere was injected with VP16-FoxD3 or FoxD3MO and embryos collected at the gastrula stage were analyzed by in situ hybridization for mesodermal, endodermal, neural and ectodermal gene expression (Fig. 5). The expression of *Brachyury*, a pan-mesodermal marker, was inhibited throughout the marginal zone by VP16-FoxD3 (Fig. 5G) and in the dorsal marginal zone by FoxD3MO (Fig. 5M). VP16-FoxD3 and FoxD3MO resulted in a near complete loss of *Chordin* and *Goosecoid*, organizer genes expressed in dorsal mesoderm (Fig. 5H,N and data not shown). The expression of *Xwnt8* in non-dorsal mesoderm was inhibited throughout the marginal zone by VP16-FoxD3 (Fig. 5I), while FoxD3MO inhibited only the dorsolateral expression of *Xwnt8* without affecting lateral and ventral expression (Fig. 5O). Pan-endodermal expression of *Mixer* and *Sox17* was unaffected by either VP16-FoxD3 or FoxD3MO (Fig. 5J,P and data not shown). *Opl* expression in the prospective neural plate was greatly reduced in response to VP16-FoxD3 and FoxD3MO (Fig. 5K,Q). Conversely, *Dlx3* expression in the non-neural ectoderm was expanded dorsally into the neural plate domain in response to VP16-FoxD3 and FoxD3MO (Fig. 5L,R). Gene expression was unaffected by the mismatch MO (Fig. 5S-X) or by a DNA-binding inactive form of VP16-

FoxD3 (data not shown). Consistent with the axial defects described above (Fig. 4), the results suggest that *FoxD3* function is required for mesoderm formation in the dorsal domain, but not for endoderm formation. Furthermore, the loss of neural plate and expansion of non-neural ectoderm is consistent with a failure to form the organizer. It should be noted that the differing extent of *Brachyury* and *Xwnt8* inhibition by VP16-FoxD3 and FoxD3MO likely reflects distinct mechanisms of FoxD3 antagonism (dominant gain-of-function versus knockdown).

Mesoderm induction by FoxD3 is non-cell-autonomous and dependent on Nodal signaling

The mesoderm-inducing activity of FoxD3 is identical to Smad2-activating members of the *TGF*β family, including the *Nodal*-related genes required for mesoderm formation (Heasman, 2006; Schier and Shen, 2000). This suggested that FoxD3 may interact with a Smad2-activating pathway to induce mesoderm, either as an upstream regulator of ligand expression, or as a downstream mediator of the response to active Smad2. To assess the potential involvement of secreted factors in the response to FoxD3, the cell autonomy of mesoderm induction by FoxD3 was examined in dissociated animal explants. In this approach, explants prepared before the midblastula transition are dissociated into individual cells in calcium-free medium to prevent a response to zygotically expressed secreted factors (Sargent et al., 1986; Wilson and Melton, 1994). Control and FoxD3-expressing animal explants were prepared at the early blastula stage (stage 7), and intact or dissociated explants were examined for mesodermal gene expression at the gastrula stage. In intact explants, FoxD3 induced expression of *Brachyury* and *MyoD*, but mesodermal gene expression was not observed in dissociated explants (Fig. 6A). To further assess the autonomy of FoxD3 function in mesoderm induction, FoxD3 RNA was injected into a single animal pole blastomere at the 32-cell stage, and the distribution of mesodermal gene expression and FoxD3 protein was examined in gastrula explants (Fig. 6B). *Brachyury* expression was induced in a ring of cells adjacent to, but not overlapping a group of cells containing nuclear FoxD3 protein. *Brachyury* mRNA and FoxD3 protein were not observed in explants of uninjected embryos (data not shown). The results indicate that FoxD3 induces mesoderm in a non-cell-autonomous manner, consistent with a role for secreted proteins in the response to FoxD3.

To assess the role of Smad2-activating pathways in FoxD3 induction of mesoderm, FoxD3 was coexpressed in animal explants with the Fast1 Smad2-interaction domain (SID), a specific inhibitor of Smad2 function (Chen et al., 1997). FoxD3 induction of *Brachyury* at the gastrula stage and of *Muscle Actin* at the tailbud stage was completely blocked by SID (Fig. 6C), indicating a requirement for a Smad2 pathway in the mesodermal response to FoxD3. The requirement for Nodal-related ligands was examined using a truncated form of Cerberus (Cerberus-short; CerS) that specifically inhibits the Nodal ligands Xnr1, Xnr2, Xnr4, Xnr5 and Xnr6 (Piccolo et al., 1999; Agius et al., 2000; Takahashi et al., 2000). Coexpression of FoxD3 and CerS resulted in a substantial reduction of *Brachyury* and a complete block of *Muscle Actin*, demonstrating that Nodal-related signals are required for the mesoderm-inducing activity of FoxD3 (Fig. 6D). The residual *Brachyury* expression suggests that there may be additional, CerS-insensitive activators of Smad2 that act together with Nodal proteins to mediate the response to FoxD3. As a positive control for inhibitory activity, SID and CerS blocked the mesoderm-inducing activity of Xnr1 (Fig. 6C,D). The results suggest that FoxD3 acts via secreted Nodal-related ligands and a Smad2 signaling pathway to induce mesoderm. Moreover, the defects in axis and mesoderm formation resulting from VP16-FoxD3 and FoxD3MO are consistent with a loss of *Nodal* function (Osada and Wright, 1999; Piccolo et al., 1999; Agius et al., 2000).

FoxD3 is necessary and sufficient for mesodermal expression of *Nodal*-related genes

The observation that FoxD3 is a non-cell autonomous, Nodal-dependent inducer of mesoderm suggests that FoxD3 regulates the expression or activity of *Nodal*-related genes. The *Xenopus*

Nodal-related genes *Xnr1*, *Xnr2*, *Xnr4*, *Xnr5* and *Xnr6* are expressed in vegetal blastomeres at the late blastula stage and in the dorsal marginal zone in the early gastrula (Jones et al., 1995; Joseph and Melton, 1997; Agius et al., 2000; Takahashi et al., 2000). At the gastrula stage, *FoxD3* is coexpressed with *Nodal*-related genes in the dorsal marginal zone (Pohl and Knochel, 2001; Sasai et al., 2001) (data not shown), suggesting that FoxD3 may regulate *Nodal* gene expression in this dorsal mesodermal domain. To assess the role of FoxD3 in regulating *Nodal*-related genes, the consequences of FoxD3 gain-of-function and knockdown on *Xnr1* and *Xnr2* expression was examined at the gastrula stage by in situ hybridization. Injection of ventral blastomeres with *FoxD3* RNA induced ectopic expression of both *Xnr1* and *Xnr2*, demonstrating that FoxD3 can promote *Nodal*-related gene expression (Fig. 7C,D). Injection of VP16-FoxD3 or FoxD3MO resulted in a loss of *Xnr1* and *Xnr2* expression in the dorsal marginal zone, indicating that FoxD3 function is required for mesodermal expression of these *Nodal*-related genes (Fig. 7E-H). Interestingly, vegetal expression of the *Nodal*-related genes, most apparent for *Xnr2* in these experiments, was unaffected by VP16-FoxD3 or FoxD3MO, suggesting that FoxD3 is not required for the vegetal endodermal expression domain (Fig. 7F,H). This result is consistent with the unperturbed expression of *Mixer* and *Sox17*, Nodal-responsive genes, in the vegetal domain of embryos injected with VP16-FoxD3 or FoxD3MO (see Fig. 5J,P and data not shown). The mismatch MO had no effect on the marginal or vegetal expression of *Xnr1* and *Xnr2* (Fig. 7I,J).

Consistent with FoxD3 induction of Nodal genes in the intact embryo, FoxD3 induced expression of *Xnr1, Xnr2* and *Xnr4* in animal explants (Fig. 7K). In addition, FoxD3 induced *Derriere*, a Smad2-activating *TGF*β family member coexpressed with *FoxD3* in the early gastrula (Sun et al., 1999). FoxD3 did not induce the expression of *Xnr5* or *Xnr6* (data not shown). To determine if FoxD3 induction of Nodal expression resulted in active signaling, phosphorylation of Smad2 was examined. In animal explants, FoxD3 induced Smad2 phosphorylation, similar to the activation of Smad2 in response to Xnr1 (Fig. 7L). Therefore, FoxD3 is necessary for the expression of *Nodal*-related genes in the organizer, and is sufficient for the induction of *Nodal*-related genes and active Nodal signaling, consistent with the embryonic defects observed with FoxD3 knockdown.

The regulation of *Nodal*-related genes by FoxD3 and the dependence of FoxD3 mesoderm-inducing activity on Nodal function suggests that *Nodal*-related genes may act downstream of FoxD3 to mediate mesoderm induction. To determine if *Nodal*-related genes function downstream of FoxD3 in the dorsal marginal zone, we attempted to rescue the axial defects resulting from FoxD3 knockdown with Xnr1. At the 4-cell stage, both dorsal blastomeres were injected with FoxD3MO alone, or in combination with *Xnr1* RNA. While most embryos were affected by injection of FoxD3MO alone (75%, n=24), coinjection of FoxD3MO and *Xnr1* resulted in a substantially reduced frequency of axial defects (24%, n=21) (Fig. 8A-E). We note that at the dose used, injection of *Xnr1* alone resulted in anterior axial defects in a minority of embryos (13%, n=23) (data not shown), consistent with previous work (Piccolo et al., 1999). In contrast to the rescue activity of Xnr1, Chordin and Dickkopf, organizer factors that regulate axis formation by inhibition of the BMP and Wnt pathways (Piccolo et al., 1996; Glinka et al., 1998), were unable to rescue FoxD3 knockdown embryos (data not shown). The interaction of FoxD3 with Xnr1 and VegT, a direct activator of Nodal expression (Kofron et al., 1999; Hyde and Old, 2000), was also examined in animal explants. Xnr1 was expressed in explants alone or in combination with FoxD3MO, and the induction of *Brachyury, MyoD, Goosecoid, Xnr1* and *Xnr2* was assessed (Fig. 8F). Mesoderm induction and Nodal autoregulation by Xnr1 was unaffected by FoxD3MO. Similarly, VegT induction of mesodermal and Nodal genes was unaffected by FoxD3MO. As controls, FoxD3MO inhibited the induction of mesodermal and Nodal genes by FoxD3, and the mismatch MO had no effect on the response to FoxD3, Xnr1 or VegT. The observation that FoxD3 knockdown did not

inhibit the activity of Xnr1 or VegT supports a role for FoxD3 as an upstream regulator of *Nodal*-related genes.

DISCUSSION

Xenopus mesoderm induction is an area of intense study that has provided fundamental insight into the molecular mechanisms of embryonic induction (Kessler, 2004; Kimelman and Bjornson, 2004). We have identified FoxD3 as an essential regulator of dorsal mesoderm formation. FoxD3 induces ectopic dorsal mesoderm and axis formation when expressed outside the Spemann organizer, and FoxD3 knockdown results in profound defects in mesodermal development and axis formation. FoxD3 is required for the expression of multiple *Nodal*-related genes in the organizer, and mesoderm induction by FoxD3 is dependent on downstream function of the Nodal signaling pathway. FoxD3 functions as a transcriptional repressor to induce *Nodal* expression and mesoderm formation, suggesting an indirect mechanism in which FoxD3 represses target gene expression to promote mesodermal development. Thus, we have identified FoxD3 as a novel regulator of mesoderm formation that prevents target gene expression in the organizer. We propose that FoxD3 functions in the Spemann organizer to repress a negative regulator of mesodermal development and maintain the expression of *Nodal*-related genes in the *Xenopus* gastrula.

FoxD3 derepression of *Nodal* expression in the Spemann Organizer

In the *Xenopus* gastrula, *FoxD3* is coexpressed with *Xnr1*, *Xnr2* and *Xnr4* in the organizer domain. The ability of FoxD3 to induce ectopic *Nodal* expression in both the marginal zone and animal pole suggests that FoxD3 is sufficient for the onset of *Nodal* gene expression. However, endogenous *FoxD3* expression lags behind the onset of *Nodal* expression in the organizer of the early gastrula, and *FoxD3* expression peaks slightly later during gastrulation (Pohl and Knochel, 2001; Sasai et al., 2001; Yaklichkin et al., 2003). Furthermore, like other organizer genes, the initiation of *Nodal*-related gene expression in the organizer is dependent on maternal VegT and nuclear ~catenin (Clements et al., 1999; Kofron et al., 1999; Agius et al., 2000; Hyde and Old, 2000; Lee et al., 2001; Xanthos et al., 2002). Taken together, the ability of regulatory inputs distinct from FoxD3 to control the onset of endogenous *Nodal* expression in the organizer and the temporal relation of *FoxD3* and *Nodal* expression suggest that FoxD3 likely functions to maintain, rather than initiate, *Nodal* expression in the organizer following the start of gastrulation.

The activity of FoxD3 fusion proteins containing a strong activation or repression domain indicates that FoxD3 functions as a transcriptional repressor to induce mesoderm. This conclusion is consistent with previous studies in cell culture and the neural crest demonstrating the repression function of FoxD3 (Sutton et al., 1996; Freyaldenhoven et al., 1997b; Pohl and Knochel, 2001; Sasai et al., 2001), and with the ability of FoxD3 to recruit Groucho corepressors and strongly repress reporter gene transcription (Yaklichkin et al., 2006). The results support a model in which FoxD3 functions as an indirect activator of *Nodal* expression by repressing a negative regulator(s) of *Nodal* in the organizer. The Nodal signaling pathway is essential for multiple aspects of vertebrate development, including induction of the endodermal and mesodermal germ layers, anterior-posterior patterning of the body axis, and establishment of left-right asymmetry (Schier and Shen, 2000; Whitman, 2001). Given these distinct roles of Nodal, it is essential that the distribution and activity of Nodal ligand, as well as the cellular response to Nodal, be precisely regulated. Misregulation of Nodal activity can result in gastrulation defects, expansion of mesodermal lineages into the ectodermal domain, loss of head structures, and situs inversus. Furthermore, since the Nodal positive feedback loop can amplify Nodal expression and signaling, mechanisms that negatively regulate Nodal expression and activity are essential for normal development.

Multiple Nodal antagonists have been identified that act at each step of the Nodal signal transduction cascade; Cerberus, Coco and Lefty/Antivin block Nodal signaling at the extracellular level (Thisse and Thisse, 1999; Piccolo et al., 1999; Cheng et al., 2000; Bell et al., 2003; Branford and Yost, 2004), while Dapper2, Smad7, Ectodermin and PIASγ act intracellularly by stimulating receptor turnover or inhibiting Smad function (Nakao et al., 1997; Casellas and Brivanlou, 1998; Daniels et al., 2004; ~hang et al., 2004b; Dupont et al., 2005). The nuclear factors Drap1, Sox3, Xema and ~ic2 inhibit the expression of *Nodal*-related genes or the transcriptional response to Nodal signals (Iratni et al., 2002; ~hang et al., 2004a; Houston and Wylie, 2005; Suri et al., 2005). These Nodal antagonists are functional in the *Xenopus* gastrula during the period of mesoderm induction and patterning, and are therefore potential regulatory targets of FoxD3.

Therefore, FoxD3 may repress antagonists that inhibit Nodal ligand-receptor interaction, inhibitors of Nodal signal transduction components, or repressors of *Nodal* transcription. While none of these potential mechanisms can be excluded at this point, we favor a role for FoxD3 in repressing a repressor of *Nodal* transcription. If FoxD3 were acting to relieve inhibition of Nodal ligand or signaling components, it is predicted that increased Nodal signaling activity would result in increased *Nodal* transcription by positive feedback. However, inhibition of Nodal ligand or signaling components, in the absence of FoxD3, would not preclude *Nodal* transcription and translation, and one might expect the accumulation of *Nodal* transcripts and protein. No *Nodal* transcripts or active Nodal signaling is detected in the animal pole (Jones et al., 1995; Joseph and Melton, 1997; Faure et al., 2000), suggesting that *Nodal* genes are maintained in an “off state” and that FoxD3 represses target genes that are required to keep *Nodal* transcriptionally silent. When ectopically expressed in the animal pole, FoxD3 is predicted to derepress *Nodal* transcription and result in robust *Nodal* expression and signaling by positive feedback. This proposed mechanism is supported by preliminary analysis of FoxD3 regulation of the *Xnr1* promoter. Basal level transcription of an *Xnr1* reporter is strongly enhanced in response to FoxD3, suggesting that FoxD3 can indirectly activate *Nodal* transcription (Q.L. and D.S.K., unpublished).

FoxD and mesodermal development in primitive chordates

In the primitive chordates *Ciona intestinalis* (ascidian) and *Branchiostoma floridae* (amphioxus), a single gene homologous to the vertebrate *FoxD* subfamily has been identified. Amphioxus *FoxD* is expressed in the dorsal mesendoderm during gastrulation, and is maintained in the axial mesendoderm and in the differentiating notochord and somites. In the amphioxus gastrula there is a striking coexpression of *FoxD* and *Nodal* in the dorsal mesendoderm (Yu et al., 2002a, 2002b). *Ciona FoxD* is expressed in the endoderm adjacent to the prospective mesoderm, and knockdown analysis indicates that FoxD is essential for the induction of mesodermal gene expression and notochord, but not for endodermal development (Imai et al., 2002). In addition, gene expression profiling of knockdown embryos indicates that FoxD is a regulator of *Nodal* expression in *Ciona*(Imai et al., 2004). These observations suggest a conserved role for *FoxD/FoxD3* genes in mesodermal development of primitive chordates and vertebrates, and this may represent the primordial developmental function for FoxD genes. We note that amphioxus and *Ciona* FoxD proteins contain a heptapeptide sequence nearly identical to the Groucho-interaction motif found in vertebrate FoxD3 proteins (S.Y. and D.S.K., unpublished), suggesting a conservation of molecular, as well as developmental function.

A *Foxd3-Nodal* connection in stem cell maintenance?

Foxd3 is expressed in the pre-implantation mouse embryo, in mouse and human embryonic stem (ES) cells, and in mouse trophoblast stem (TS) cells (Sutton et al., 1996; Pera et al., 2000; Hanna et al., 2002; Tompers et al., 2005). At the gastrula stage, *Foxd3* is expressed uniformly in the epiblast, including cells of the node, and in scattered cells of the

extraembryonic ectoderm. *Foxd3* null embryos die at 6.5 dpc with a loss of epiblast cells and an expansion of extraembryonic tissues. Null embryos do not initiate gastrulation, fail to form mesoderm, and do not express *Nodal* in the epiblast, but due to the early epiblast defect it not yet clear if FoxD3 is specifically required for mesoderm formation in the mouse. In chimeras, a small contribution of wild-type cells can rescue null embryos, suggesting that FoxD3 function in the epiblast is non-cell autonomous. In culture, the inner cell mass of null embryos initially proliferates but is not maintained, and FoxD3 null ES cell lines cannot be established (Hanna et al., 2002). *FoxD3* is also essential for normal placental development, and the trophoblast progenitors of null embryos do not self-renew and are not multipotent (Tompers et al., 2005).

The interaction of FoxD3 and Nodal in *Xenopus* mesoderm formation raises the possibility that there is an interaction between FoxD3 and Nodal in stem cell maintenance. In fact, *Nodal*is required to maintain the TS cell compartment in the mouse embryo, and Nodal protein maintains the pluripotency of human ES cells in culture (Besser, 2004; Guzman-Ayala et al., 2004; Vallier et al., 2004, 2005; James et al., 2005). These results suggest that *Nodal*, like *FoxD3*, is essential for stem cell maintenance. However, *Nodal* null ES cell lines can be established at expected frequencies, arguing against a requirement for Nodal function in maintaining mouse ES cells (Conlon et al., 1991). These apparently contradictory results may reflect the ability of Nodal protein to mimic a distinct TGFβ ligand or, alternatively, that Nodal may function redundantly with other TGFβ ligands to maintain ES cells. Two additional TGFβ family members, *Gdf1* and *Gdf3*, are expressed in the early mouse embryo before or just after implantation, and both are identical to Nodal in signaling activity (Jones et al., 1992; McPherron and Lee, 1993; Rankin et al., 2000; Cheng et al., 2003; Chen et al., 2006; Levine and Brivanlou, 2006). Genetic analyses have demonstrated a synergistic interaction between *Nodal* and *Gdf1* in early mouse development (Andersson et al., 2006). *Gdf3* is expressed in mouse and human ES cells and maintains markers of pluripotency in cultured ES cells (Clark et al., 2004; Levine and Brivanlou, 2006). Whether *Nodal*, *Gdf1* and *Gdf3* contribute to embryonic stem cell maintenance in vivo, and whether *FoxD3* functionally interacts with these putative maintenance factors are significant questions for further study.

FoxD3 has a demonstrated role in multiple processes of vertebrate development. Among the many remaining questions to explore, it will be important to identify the transcriptional targets of FoxD3 that mediate its distinct embryonic functions. Whether similar sets of FoxD3 target genes are identified in different contexts will reveal if a common regulatory pathway is utilized in each of these lineages, or if there are lineage-specific mechanisms of FoxD3 function. Ongoing studies of FoxD3 in the organizer, the neural crest, and stem cell populations are likely to provide further insight into the developmental and molecular mechanisms of vertebrate embryogenesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We are grateful to Steve DiNardo, Doug Epstein and Peter Klein for critical reading of the manuscript. We thank Eddy De Robertis, Peter Klein, Stefano Piccolo, Jean-Pierre Saint-Jeannet, Yoshiki Sasai, Hazel Sive, Malcolm Whitman and Chris Wright for providing plasmids. We further thank Malcolm Whitman for helpful advice in the analysis of phosphoSmad2. We also thank Jacquie Kolezki and Valerie Cluzet for contributing to the initial experiments. This work was supported by grants from the NIH (HD36720) and the American Heart Association to P.A.L., and by grants from the NIH (GM64768) and the Pew Scholars Program in the Biomedical Sciences to D.S.K.

REFERENCES

Agius E, Oelgeschlager M, Wessely O, Kemp C, De Robertis EM. Endodermal Nodal-related signals and mesoderm induction in *Xenopus*. Development 2000;127:1173–1183. [PubMed: 10683171]

Alkhateeb A, Fain PR, Spritz RA. Candidate functional promoter variant in the FOXD3 melanoblast developmental regulator gene in autosomal dominant vitiligo. J. Invest. Dermatol 2005;125:388–391. [PubMed: 16098053]

Andersson O, Reissmann E, Jornvall H, Ibanez CF. Synergistic interaction between Gdf1 and Nodal during anterior axis development. Dev. Biol 2006;293:370–381. [PubMed: 16564040]

Badiani P, Corbella P, Kioussis D, Marvel J, Weston K. Dominant interfering alleles define a role for c-Myb in T-cell development. Genes Dev 1994;8:770–782. [PubMed: 7926766]

Bell E, Munoz-Sanjuan I, Altmann CR, Vonica A, Brivanlou AH. Cell fate specification and competence by Coco, a maternal BMP, TGF-beta and Wnt inhibitor. Development 2003;130:1381–1389. [PubMed: 12588853]

Besser D. Expression of nodal, lefty-a, and lefty-B in undifferentiated human embryonic stem cells requires activation of Smad2/3. J. Biol. Chem 2004;279:45076–45084. [PubMed: 15308665]

Bolce ME, Hemmati-Brivanlou A, Kushner PD, Harland RM. Ventral ectoderm of *Xenopus* forms neural tissue, including hindbrain, in response to activin. Development 1992;115:681–688. [PubMed: 1425347]

Branford WW, Yost HJ. Nodal signaling: Cryptic Lefty mechanism of antagonism decoded. Curr. Biol 2004;14:R341–343. [PubMed: 15120085]

Carlsson P, Mahlapuu M. Forkhead transcription factors: key players in development and metabolism. Dev. Biol 2002;250:1–23. [PubMed: 12297093]

Casellas R, Brivanlou AH. *Xenopus* Smad7 inhibits both the activin and BMP pathways and acts as a neural inducer. Dev. Biol 1998;198:1–12. [PubMed: 9640328]

Chen X, Weisberg E, Fridmacher V, Watanabe M, Naco G, Whitman M. Smad4 and FAST-1 in the assembly of activin-responsive factor. Nature 1997;389:85–89. [PubMed: 9288972]

Chen C, Ware SM, Sato A, Houston-Hawkins DE, Habas R, Matzuk MM, Shen MM, Brown CW. The Vg1-related protein Gdf3 acts in a Nodal signaling pathway in the pre-gastrulation mouse embryo. Development 2006;133:319–329. [PubMed: 16368929]

Cheng AM, Thisse B, Thisse C, Wright CV. The lefty-related factor Xatv acts as a feedback inhibitor of nodal signaling in mesoderm induction and L-R axis development in xenopus. Development 2000;127:1049–1061. [PubMed: 10662644]

Cheng SK, Olale F, Bennett JT, Brivanlou AH, Schier AF. EGF-CFC proteins are essential coreceptors for the TGF-beta signals Vg1 and GDF1. Genes Dev 2003;17:31–36. [PubMed: 12514096]

Cheung M, Chaboissier MC, Mynett A, Hirst E, Schedl A, Briscoe J. The transcriptional control of trunk neural crest induction, survival, and delamination. Dev. Cell 2005;8:179–192. [PubMed: 15691760]

Cho KW, Blumberg B, Steinbeisser H, De Robertis EM. Molecular nature of Spemann's organizer: the role of the *Xenopus* homeobox gene goosecoid. Cell 1991;67:1111–1120. [PubMed: 1684739]

Clark AT, Rodriguez RT, Bodnar MS, Abeyta MJ, Cedars MI, Turek PJ, Firpo MT, Reijo Pera RA. Human STELLAR, NANOG, and GDF3 genes are expressed in pluripotent cells and map to chromosome 12p13, a hotspot for teratocarcinoma. Stem Cells 2004;22:169–179. [PubMed: 14990856]

Clements D, Friday RV, Woodland HR. Mode of action of VegT in mesoderm and endoderm formation. Development 1999;126:4903–4911. [PubMed: 10518506]

Conlon FL, Barth KS, Robertson EJ. A novel retrovirally induced embryonic lethal mutation in the mouse: assessment of the developmental fate of embryonic stem cells homozygous for the 413.d proviral integration. Development 1991;111:969–981. [PubMed: 1879365]

Conlon FL, Sedgwick SG, Weston KM, Smith JC. Inhibition of Xbra transcription activation causes defects in mesodermal patterning and reveals autoregulation of Xbra in dorsal mesoderm. Development 1996;122:2427–2435. [PubMed: 8756288]

Daniels M, Shimizu K, Zorn AM, Ohnuma S. Negative regulation of Smad2 by PIASy is required for proper *Xenopus* mesoderm formation. Development 2004;131:5613–5626. [PubMed: 15496439]

De Robertis EM, Larrain J, Oelgeschlager M, Wessely O. The establishment of Spemann's organizer and patterning of the vertebrate embryo. Nature Rev. Genet 2000;1:171–181. [PubMed: 11252746]

Dirksen ML, Jamrich M. Differential expression of fork head genes during early *Xenopus* and zebrafish development. Dev. Genet 1995;17:107–116. [PubMed: 7586752]

Dottori M, Gross MK, Labosky P, Goulding M. The winged-helix transcription factor Foxd3 suppresses interneuron differentiation and promotes neural crest cell fate. Development 2001;128:4127–4138. [PubMed: 11684651]

Dupont S, Zacchigna L, Cordenonsi M, Soligo S, Adorno M, Rugge M, Piccolo S. Germ-layer specification and control of cell growth by Ectodermin, a Smad4 ubiquitin ligase. Cell 2005;121:87–99. [PubMed: 15820681]

Engleka MJ, Craig EJ, Kessler DS. VegT activation of Sox17 at the midblastula transition alters the response to Nodal signals in the vegetal endoderm domain. Dev. Biol 2001;237:159–172. [PubMed: 11518513]

Faure S, Lee MA, Keller T, ten Dijke P, Whitman M. Endogenous patterns of TGFβ superfamily signaling during early *Xenopus* development. Development 2000;127:2917–2931. [PubMed: 10851136]

Feledy JA, Beanan MJ, Sandoval JJ, Goodrich JS, Lim JH, Matsuo-Takasaki M, Sato SM, Sargent TD. Inhibitory patterning of the anterior neural plate in *Xenopus* by homeodomain factors Dlx3 and Msx1. Dev. Biol 1999;212:455–464. [PubMed: 10433834]

Freyaldenhoven BS, Freyaldenhoven MP, Iacovoni JS, Vogt PK. Aberrant cell growth induced by avian winged helix proteins. Cancer Res 1997a;57:123–129. [PubMed: 8988052]

Freyaldenhoven BS, Freyaldenhoven MP, Iacovoni JS, Vogt PK. Avian winged helix proteins CWH-1, CWH-2 and CWH-3 repress transcription from Qin binding sites. Oncogene 1997b;15:483–488. [PubMed: 9242385]

Glinka A, Wu W, Delius H, Monaghan AP, Blumenstock C, Niehrs C. Dickkopf-1 is a member of a new family of secreted proteins and functions in head induction. Nature 1998;391:357–362. [PubMed: 9450748]

Guzman-Ayala M, Ben-Haim N, Beck S, Constam DB. Nodal protein processing and fibroblast growth factor 4 synergize to maintain a trophoblast stem cell microenvironment. Proc. Natl. Acad. Sci. USA 2004;101:15656–15660. [PubMed: 15505202]

Han K, Manley JL. Functional domains of the Drosophila Engrailed protein. EMBO J 1993;12:2723–2733. [PubMed: 8334991]

Hanna LA, Foreman RK, Tarasenko IA, Kessler DS, Labosky PA. Requirement for Foxd3 in maintaining pluripotent cells of the early mouse embryo. Genes Dev 2002;16:2650–2661. [PubMed: 12381664]

Harland R, Gerhart J. Formation and function of Spemann's organizer. Annu. Rev. Cell Dev. Biol 1997;13:611–667. [PubMed: 9442883]

Heasman J. Patterning the early Xenopus embryo. Development 2006;133:1205–1217. [PubMed: 16527985]

Heasman J, Kofron M, Wylie C. β-Catenin signaling activity dissected in the early *Xenopus* embryo: a novel antisense approach. Dev. Biol 2000;222:124–134. [PubMed: 10885751]

Houston DW, Wylie C. Maternal *Xenopus* ~ic2 negatively regulates Nodal-related gene expression during anteroposterior patterning. Development 2005;132:4845–4855. [PubMed: 16207750]

Hyde CE, Old RW. Regulation of the early expression of the *Xenopus* nodal-related 1 gene, Xnr1. Development 2000;127:1221–1229. [PubMed: 10683175]

Imai KS, Satoh N, Satou Y. An essential role of a FoxD gene in notochord induction in Ciona embryos. Development 2002;129:3441–3453. [PubMed: 12091314]

Imai KS, Hino K, Yagi K, Satoh N, Satou Y. Gene expression profiles of transcription factors and signaling molecules in the ascidian embryo: towards a comprehensive understanding of gene networks. Development 2004;131:4047–4058. [PubMed: 15269171]

Iratni R, Yan YT, Chen C, Ding J, Zhang Y, Price SM, Reinberg D, Shen MM. Inhibition of excess nodal signaling during mouse gastrulation by the transcriptional corepressor DRAP1. Science 2002;298:1996–1999. [PubMed: 12471260]

James D, Levine AJ, Besser D, Hemmati-Brivanlou A. TGFbeta/activin/nodal signaling is necessary for the maintenance of pluripotency in human embryonic stem cells. Development 2005;132:1273–1282. [PubMed: 15703277]

Jaynes JB, O'Farrell PH. Active repression of transcription by the engrailed homeodomain protein. EMBO J 1991;10:1427–1433. [PubMed: 1673924]

Jones CM, Kuehn MR, Hogan BL, Smith JC, Wright CV. Nodal-related signals induce axial mesoderm and dorsalize mesoderm during gastrulation. Development 1995;121:3651–3662. [PubMed: 8582278]

Jones CM, Simon-Chazottes D, Guenet JL, Hogan BL. Isolation of Vgr-2, a novel member of the transforming growth factor-beta-related gene family. Mol. Endocrinol 1992;6:1961–1968. [PubMed: 1480182]

Joseph EM, Melton DA. Xnr4: a *Xenopus* nodal-related gene expressed in the Spemann organizer. Dev. Biol 1997;184:367–372. [PubMed: 9133442]

Kelsh RN, Dutton K, Medlin J, Eisen JS. Expression of zebrafish fkd6 in neural crest-derived glia. Mech. Dev 2000;93:161–164. [PubMed: 10781949]

Kessler DS. Siamois is required for formation of Spemann's organizer. Proc. Natl. Acad. Sci. USA 1997;94:13017–13022. [PubMed: 9371792]

Kessler, DS. Activin and Vg1 and the Search for Embryonic Inducers. In: Stern, CD., editor. Gastrulation: From Cells to Embryo. Cold Spring Harbor Laboratory Press; Cold Spring Harbor: 2004. p. 505-520.

Kimelman, D.; Bjornson, C. Vertebrate Mesoderm Induction. In: Stern, CD., editor. Gastrulation: From Cells to Embryos. Cold Spring Harbor Laboratory Press; Cold Spring Harbor: 2004. p. 363-372.

Kintner CR, Brockes JP. Monoclonal antibodies identify blastemal cells derived from dedifferentiating muscle in newt limb regeneration. Nature 1984;308:67–69. [PubMed: 6366572]

Klein SL. The first cleavage furrow demarcates the dorsal-ventral axis in *Xenopus* embryos. Dev. Biol 1987;120:299–304. [PubMed: 3817297]

Kofron M, Demel T, Xanthos J, Lohr J, Sun B, Sive H, Osada S, Wright C, Wylie C, Heasman J. Mesoderm induction in *Xenopus* is a zygotic event regulated by maternal VegT via TGF-beta growth factors. Development 1999;126:5759–5770. [PubMed: 10572051]

Kos R, Reedy MV, Johnson RL, Erickson CA. The winged-helix transcription factor FoxD3 is important for establishing the neural crest lineage and repressing melanogenesis in avian embryos. Development 2001;128:1467–1479. [PubMed: 11262245]

Kuo JS, Patel M, Gamse J, Merzdorf C, Liu X, Apekin V, Sive H. Opl: a zinc finger protein that regulates neural determination and patterning in *Xenopus*. Development 1998;125:2867–2882. [PubMed: 9655809]

Labosky PA, Kaestner KH. The winged helix transcription factor Hfh2 is expressed in neural crest and spinal cord during mouse development. Mech. Dev 1998;76:185–190. [PubMed: 9767163]

Lee MA, Heasman J, Whitman M. Timing of endogenous activin-like signals and regional specification of the *Xenopus* embryo. Development 2001;128:2939–2952. [PubMed: 11532917]

Lef J, Dege P, Scheucher M, Forsbach-Birk V, Clement JH, Knochel W. A fork head related multigene family is transcribed in *Xenopus laevis* embryos. Int. J. Dev. Biol 1996;40:245–253. [PubMed: 8735935]

Lehmann OJ, Sowden JC, Carlsson P, Jordan T, Bhattacharya SS. Fox's in development and disease. Trends Genet 2003;19:339–344. [PubMed: 12801727]

Levine AJ, Brivanlou AH. GDF3, a BMP inhibitor, regulates cell fate in stem cells and early embryos. Development 2006;133:209–216. [PubMed: 16339188]

Lister JA, Cooper C, Nguyen K, Modrell M, Grant K, Raible DW. ~ebrafish Foxd3 is required for development of a subset of neural crest derivatives. Dev. Biol 2006;290:92–104. [PubMed: 16364284]

McPherron AC, Lee SJ. GDF-3 and GDF-9: two new members of the transforming growth factor-beta superfamily containing a novel pattern of cysteines. J. Biol. Chem 1993;268:3444–3449. [PubMed: 8429021]

Nakao A, Afrakhte M, Moren A, Nakayama T, Christian JL, Heuchel R, Itoh S, Kawabata M, Heldin NE, Heldin CH, ten Dijke P. Identification of Smad7, a TGF-beta-inducible antagonist of TGF-beta signalling. Nature 1997;389:631–635. [PubMed: 9335507]

Nieuwkoop, PD.; Faber, J. Normal Table of Xenopus laevis (Daudin). North Holland Publishing Company; Amsterdam: 1967.

Odenthal J, Nusslein-Volhard C. fork head domain genes in zebrafish. Dev. Genes Evol 1998;208:245–258. [PubMed: 9683740]

Osada SI, Wright CV. *Xenopus* nodal-related signaling is essential for mesendodermal patterning during early embryogenesis. Development 1999;126:3229–3240. [PubMed: 10375512]

Pera MF, Reubinoff B, Trounson A. Human embryonic stem cells. J. Cell Sci 2000;113:5–10. [PubMed: 10591620]

Piccolo S, Sasai Y, Lu B, De Robertis EM. Dorsoventral patterning in Xenopus: inhibition of ventral signals by direct binding of chordin to BMP-4. Cell 1996;86:589–598. [PubMed: 8752213]

Piccolo S, Agius E, Leyns L, Bhattacharyya S, Grunz H, Bouwmeester T, De Robertis EM. The head inducer Cerberus is a multifunctional antagonist of Nodal, BMP and Wnt signals. Nature 1999;397:707–710. [PubMed: 10067895]

Pohl BS, Knochel W. Overexpression of the transcriptional repressor FoxD3 prevents neural crest formation in *Xenopus* embryos. Mech. Dev 2001;103:93–106. [PubMed: 11335115]

Pohl BS, Knochel W. Of Fox and Frogs: Fox (fork head/winged helix) transcription factors in *Xenopus* development. Gene 2005;344:21–32. [PubMed: 15656969]

Rankin CT, Bunton T, Lawler AM, Lee SJ. Regulation of left-right patterning in mice by growth/differentiation factor-1. Nat. Genet 2000;24:262–265. [PubMed: 10700179]

Rupp RA, Snider L, Weintraub H. *Xenopus* embryos regulate the nuclear localization of XMyoD. Genes Dev 1994;8:1311–1323. [PubMed: 7926732]

Sadowski I, Ma J, Triezenberg S, Ptashne M. GAL4-VP16 is an unusually potent transcriptional activator. Nature 1988;335:563–564. [PubMed: 3047590]

Sampath K, Cheng AM, Frisch A, Wright CV. Functional differences among *Xenopus* nodal-related genes in left-right axis determination. Development 1997;124:3293–3302. [PubMed: 9310324]

Sargent TD, Jamrich M, Dawid IB. Cell interactions and the control of gene activity during early development of *Xenopus laevis*. Dev. Biol 1986;114:238–246. [PubMed: 3956863]

Sasai N, Mizuseki K, Sasai Y. Requirement of FoxD3-class signaling for neural crest determination in *Xenopus*. Development 2001;128:2525–2536. [PubMed: 11493569]

Sasai Y, Lu B, Steinbeisser H, Geissert D, Gont LK, De Robertis EM. *Xenopus* chordin: a novel dorsalizing factor activated by organizer- specific homeobox genes. Cell 1994;79:779–790. [PubMed: 8001117]

Scheucher M, Dege P, Lef J, Hille S, Knochel W. Transcription patterns of four different *forkhead*/HNF-3 related genes (XFD-4, 6, 9 and 10) in *Xenopus laevis* embryos. Roux's Arch. Dev. Biol 1995;204:203–211.

Schier AF, Shen MM. Nodal signalling in vertebrate development. Nature 2000;403:385–389. [PubMed: 10667782]

Sive, HL.; Grainger, RM.; Harland, RM. Early development of Xenopus laevis: a laboratory manual. CSHL Press; Cold Spring Harbor: 2000.

Sokol S, Christian JL, Moon RT, Melton DA. Injected wnt RNA induces a complete body axis in *Xenopus* embryos. Cell 1991;67:741–752. [PubMed: 1834344]

Stewart RA, Arduini BL, Berghmans S, George RE, Kanki JP, Henion PD, Look AT. ~ebrafish foxd3 is selectively required for neural crest specification, migration and survival. Dev. Biol 2006;292:174–188. [PubMed: 16499899]

Summerton J, Weller D. Morpholino antisense oligomers: design, preparation, and properties. Antisense Nuc. Acid Drug Dev 1997;7:187–195.

Sun BI, Bush SM, Collins-Racie LA, LaVallie ER, DiBlasio-Smith EA, Wolfman NM, McCoy JM, Sive HL. derriere: a TGF-beta family member required for posterior development in *Xenopus*. Development 1999;126:1467–1482. [PubMed: 10068640]

Suri C, Haremaki T, Weinstein DC. Xema, a *foxi*-class gene expressed in the gastrula stage *Xenopus* ectoderm, is required for the suppression of mesendoderm. Development 2005;132:2733–2742. [PubMed: 15901660]

Sutton J, Costa R, Klug M, Field L, Xu D, Largaespada DA, Fletcher CF, Jenkins NA, Copeland NG, Klemsz M, Hromas R. Genesis, a winged helix transcriptional repressor with expression restricted to embryonic stem cells. J. Biol. Chem 1996;271:23126–23133. [PubMed: 8798505]

Symes K, Smith JC. Gastrulation movements provide an early marker of mesoderm induction in *Xenopus laevis*. Development 1987;101:339–349.

Takahashi S, Yokota C, Takano K, Tanegashima K, Onuma Y, Goto JI, Asashima M. Two novel nodal-related genes initiate early inductive events in *Xenopus* Nieuwkoop center. Development 2000;127:5319–5329. [PubMed: 11076754]

Thisse C, Thisse B. Antivin, a novel and divergent member of the TGF-beta superfamily, negatively regulates mesoderm induction. Development 1999;126:229–240. [PubMed: 9847237]

Tompers DM, Foreman RK, Wang Q, Kumanova M, Labosky PA. Foxd3 is required in the trophoblast progenitor cell lineage of the mouse embryo. Dev. Biol 2005;285:126–137. [PubMed: 16039639]

Triezenberg SJ, Kingsbury RC, McKnight SL. Functional dissection of VP16, the trans-activator of herpes simplex virus immediate early gene expression. Genes Dev 1988;2:718–729. [PubMed: 2843425]

Vallier L, Reynolds D, Pedersen RA. Nodal inhibits differentiation of human embryonic stem cells along the neuroectodermal default pathway. Dev. Biol 2004;275:403–421. [PubMed: 15501227]

Vallier L, Alexander M, Pedersen RA. Activin/Nodal and FGF pathways cooperate to maintain pluripotency of human embryonic stem cells. J. Cell Sci 2005;118:4495–4509. [PubMed: 16179608]

Watanabe M, Frelinger AL, Rutishauser U. Topography of N-CAM structural and functional determinants. I. Classification of monoclonal antibody epitopes. J. Cell Biol 1986;103:1721–1727. [PubMed: 2430976]

Whitlock KE, Smith KM, Kim H, Harden MV. A role for foxd3 and sox10 in the differentiation of gonadotropin-releasing hormone (GnRH) cells in the zebrafish Danio rerio. Development 2005;132:5491–5502. [PubMed: 16291787]

Whitman M. Nodal signaling in early vertebrate embryos: themes and variations. Dev. Cell 2001;1:605–617. [PubMed: 11709181]

Wilson PA, Melton DA. Mesodermal patterning by an inducer gradient depends on secondary cell-cell communication. Curr. Biol 1994;4:676–686. [PubMed: 7953553]

Xanthos JB, Kofron M, Tao Q, Schaible K, Wylie C, Heasman J. The roles of three signaling pathways in the formation and function of the Spemann Organizer. Development 2002;129:4027–4043. [PubMed: 12163406]

Yaklichkin, S.; Steiner, AB.; Kessler, DS. Transcriptional repression in Spemann's Organizer and the formation of dorsal mesoderm. In: Grunz, H., editor. The Vertebrate Organizer. Springer-Verlag Press; Heidelberg: 2003. p. 113-126.

Yaklichkin S, Steiner AB, Kessler DS. FoxD3 and Grg4 physically interact to repress transcription and induce mesoderm in *Xenopus*. Journal of Biological Chemistry. 2006in press

Yamagata M, Noda M. The winged-helix transcription factor CWH-3 is expressed in developing neural crest cells. Neurosci. Lett 1998;249:33–36. [PubMed: 9672382]

Yao, J.; Kessler, DS. Mesoderm induction in *Xenopus*: oocyte expression system and animal cap assay. In: Tuan, RS.; Lo, CW., editors. Methods in Molecular Biology, Vol. 137: Developmental Biology Protocols, Vol. III. Humana Press; Totowa: 1999. p. 576-585.

Yu JK, Holland LZ, Holland ND. An amphioxus nodal gene (AmphiNodal) with early symmetrical expression in the organizer and mesoderm and later asymmetrical expression associated with left-right axis formation. Evol. Dev 2002a;4:418–425. [PubMed: 12492142]

Yu JK, Holland ND, Holland LZ. An amphioxus winged helix/forkhead gene, AmphiFoxD: insights into vertebrate neural crest evolution. Dev. Dyn 2002b;225:289–297. [PubMed: 12412011]

Zhang C, Basta T, Hernandez-Lagunas L, Simpson P, Stemple DL, Artinger KB, Klymkowsky MW. Repression of nodal expression by maternal B1-type SOXs regulates germ layer formation in *Xenopus* and zebrafish. Dev. Biol 2004a;273:23–37. [PubMed: 15302595]

Zhang L, Zhou H, Su Y, Sun Z, Zhang H, Zhang Y, Ning Y, Chen YG, Meng A. ~ebrafish Dpr2 inhibits mesoderm induction by promoting degradation of nodal receptors. Science 2004b;306:114–117. [PubMed: 15459392]

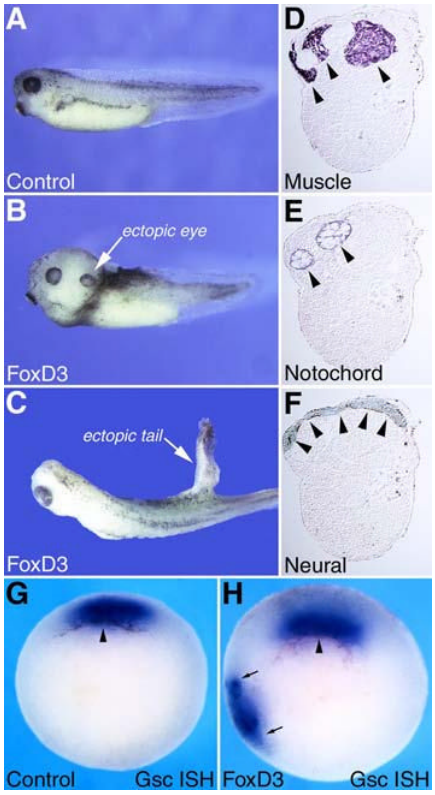

Figure 1.

Ectopic axis induction by FoxD3. At the 4-cell stage a single ventral blastomere was injected with *FoxD3*RNA (100 or 300 pg). Ectopic anterior axial structures, including ectopic eyes, were induced at the high dose (**B**) and ectopic tails were induced at the low dose (**C**). Embryos were analyzed at stage 35 by serial-section immunocytochemistry to detect muscle (12/101) (**D**), notochord (Tor70) (**E**) and neural tube (4d) (**F**) (transverse sections, dorsal up; arrowheads indicate stained tissues). Embryos were also analyzed at the early gastrula stage (stage 10.25) by whole-mount in situ hybridization for the expression of *Goosecoid* (**G,H**). FoxD3 induced ectopic *Goosecoid* expression (**H**) (vegetal views, dorsal up; arrowheads indicate dorsal blastopore lip and arrows indicate region of ectopic gene expression).

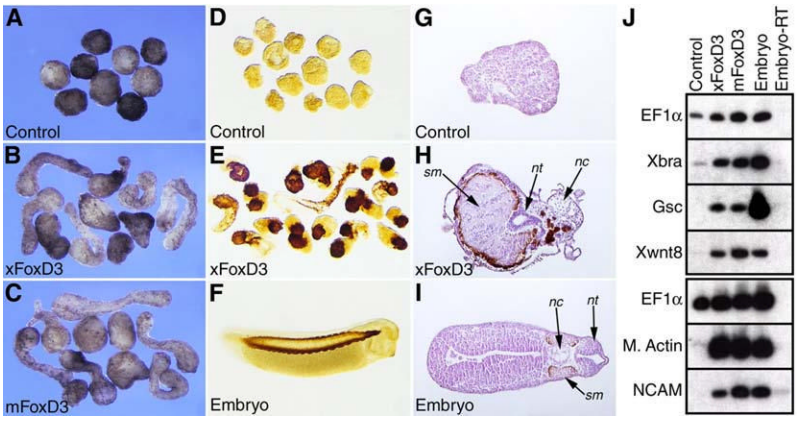

Figure 2.

Mesoderm induction by FoxD3. At the one-cell stage, embryos were injected in the animal pole with 200 pg of *Xenopus FoxD3* (xFoxD3) or mouse *FoxD3* (mFoxD3), explants were prepared at the late blastula stage (stage 9), and explants were analyzed for morphogenesis, tissue differentiation and gene expression. At the tailbud stage (stage 25), convergent extension movements were observed in response to xFoxD3 and mFoxD3 (**A-C**), and differentiated somitic muscle was detected in the FoxD3-expressing explants (**D-F**) using a muscle-specific antibody (12/101). (**G-I**) Explants stained with 12/101 were sectioned and counterstained (H&E) to show the presence of somitic muscle (sm), notochord (nc) and neural tube (nt) in FoxD3-induced explants. (**J**) Gene expression in explants was examined by RT-PCR for *Brachyury* (Xbra), *Goosecoid* (Gsc) and *Xwnt8* at the midgastrula stage (stage 11), and for *Muscle Actin* (M. Actin) and *NCAM* at the tailbud stage (stage 25). *EF1α* is a control for RNA recovery and loading, intact embryos (Embryo) served as a positive control and an identical reaction without reverse transcriptase controlled for PCR contamination (Embryo-RT).

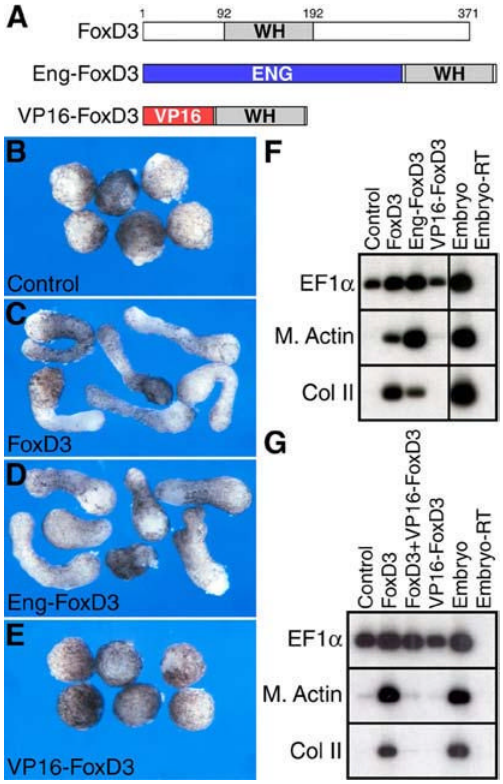

Figure 3.

Functional analysis of FoxD3 fusion proteins. (**A**) Schematic of the structure of FoxD3 and the FoxD3 fusion proteins. FoxD3 contains a conserved winged helix (WH) DNA-binding domain (residues 92-192). The repressor fusion protein (Eng-FoxD3) contains the repressor domain of *Drosophila* Engrailed (residues 1-298) fused to the FoxD3 WH domain. The activator fusion protein (VP16-FoxD3) contains the activation domain of HSV VP16 (residues 410-490). Embryos were injected with FoxD3 (100 pg), Eng-FoxD3 (100 pg) or VP16-FoxD3 (250 pg) and animal explants were analyzed at the tailbud stage (stage 25) for morphogenesis (**B-E**) and by RT-PCR for the expression of *Muscle Actin* (M. Actin) and *Collagen Type II* (Col II) (**F**). Like FoxD3, Eng-FoxD3 induced convergent extension movements and mesodermal gene expression, while VP16-FoxD3 did not. (**G**) Coexpression of VP16-FoxD3 and FoxD3 blocked induction of mesodermal genes by FoxD3. PCR controls are as described in Fig. 2.

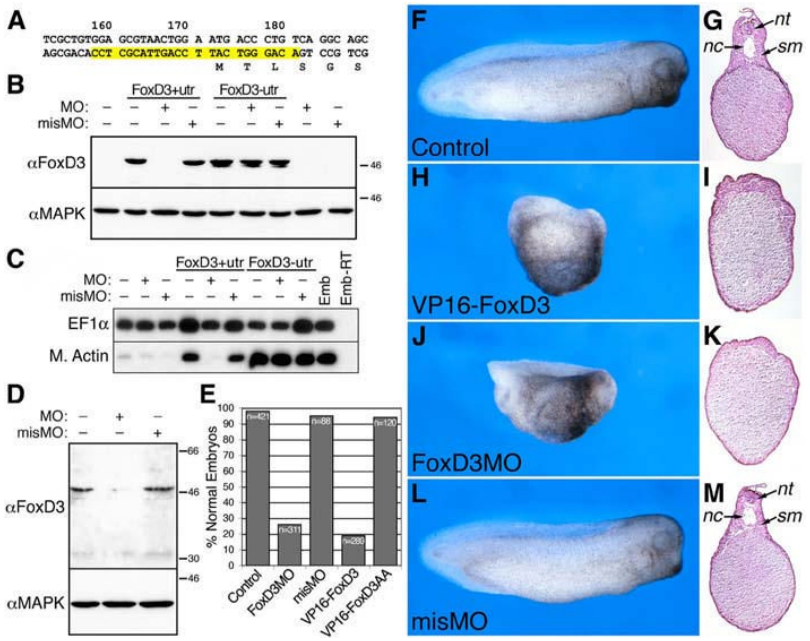

Figure 4.

FoxD3 function is required for axis formation. (**A**) The sequence of *FoxD3* flanking the initiator methionine with the sequence of the morpholino antisense oligonucleotide (181-158) highlighted in yellow. (**B**) Western analysis of animal explants prepared from embryos injected with *FoxD3* RNAs (2 ng) alone, or in combination with antisense (FoxD3MO) or mismatch (misMO) morpholino oligonucleotides (50 ng). Translation of a *FoxD3* RNA containing the 5′ UTR and the complete antisense target sequence (FoxD3+utr) was inhibited by FoxD3MO, but not misMO. Translation of *FoxD3* lacking the 5′ UTR (FoxD3-utr) was unaffected by either oligonucleotide. Equal protein loading was confirmed by blotting for the ubiquitous MAPK. (**C**) RT-PCR analysis of *Muscle Actin* (M. Actin) induction in animal explants injected with *FoxD3* RNAs containing or lacking the 5′ UTR (200 pg) and FoxD3MO or misMO (50 ng). PCR controls are as described in Fig. 2. (**D**) At the four-cell stage each blastomere was injected in the marginal zone with FoxD3MO or misMO (25 ng), and extracts prepared at the mid-gastrula stage (stage 11) were analyzed by western blotting for the accumulation of endogenous FoxD3 protein. A single major band, migrating at the same position as overexpressed *Xenopus* FoxD3, was detected in uninjected and misMO-injected samples, and was reduced ~10-fold in FoxD3MO-injected samples. The exposure of the western blot in panel D was approximately eight times longer than that shown in panel B. (**E-M**) At the four-cell stage each blastomere was injected in the marginal zone with 250 pg of VP16-FoxD3 RNA (**H,I**), 15 ng of FoxD3MO (**J,K**) or 15 ng of misMO (**L,M**). At the tailbud stage (stage 30), embryos were sectioned (transverse, dorsal up) to examine the formation of axial structures, including notochord (nc), somitic muscle (sm) and neural tube (nt) (**G,I,K,M**). (**E**) Quantification of the combined results of five independent experiments.

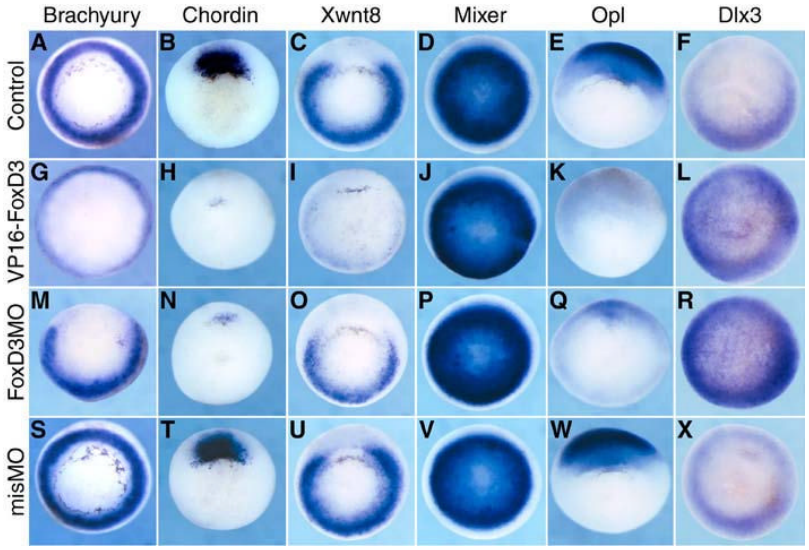

Figure 5.

Mesodermal gene expression is dependent on FoxD3 function. At the four-cell stage, each blastomere was injected in the marginal zone with 500 pg of VP16-FoxD3 RNA (**G-L**), 25 ng of FoxD3MO (**M-R**), or 25 ng of mismatch MO (**S-X**). At the early gastrula stage (stage 10.25), embryos were analyzed by in situ hybridization for the expression of the indicated genes. The results shown are representative of three independent experiments (n=12-18 embryos per sample in each experiment). Vegetal views are shown for *Brachyury*, *Chordin*, *Xwnt8*, *Mixer* and *Opl*, animal views are shown for *Dlx3*, and dorsal is up for all panels.

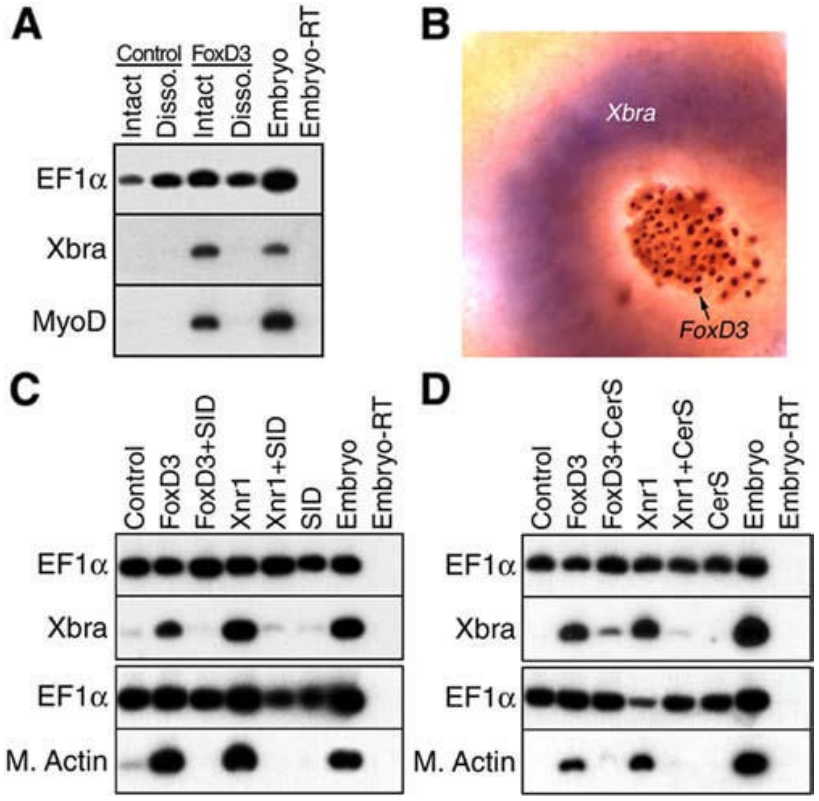

Figure 6.

Mesoderm induction by FoxD3 is non-cell-autonomous and dependent on the Nodal pathway. (**A**) At the one-cell stage the animal pole was injected with 100 pg of FoxD3 RNA and animal explants prepared at the early blastula (stage 7) were cultured intact or dissociated into individual cells in the absence of calcium (Dissoc.). The expression of *Brachyury* (Xbra), and *MyoD* was examined in uninjected (Control) and injected explants by RT-PCR at the gastrula stage (stage 11). (**B**) At the 32-cell stage a single animal pole blastomere was injected with 100 pg of FoxD3 RNA and explants prepared and fixed at the early gastrula stage (stage 10.5) were sequentially examined for *Brachyury* (Xbra) expression by in situ hybridization and FoxD3 protein expression by immunocytochemistry. To assess the dependence of FoxD3 function on Smad2 and Nodal, FoxD3 (100 pg) was injected alone, or in combination with 1 ng of the Smad2-interaction domain of Fast1 (SID) (**C**) or 1 ng of a truncated form of Cerberus (CerS) (**D**). Animal explants prepared at the midblastula stage (stage 9) were collected for RT-PCR analysis of *Brachyury* (Xbra) at the gastrula stage (stage 11) and *Muscle Actin* (M. Actin) at the tailbud stage (stage 25). Xnr1 (50 pg) was used as a positive control for the inhibitory activity of SID and CerS. PCR controls are as described in Fig. 2.

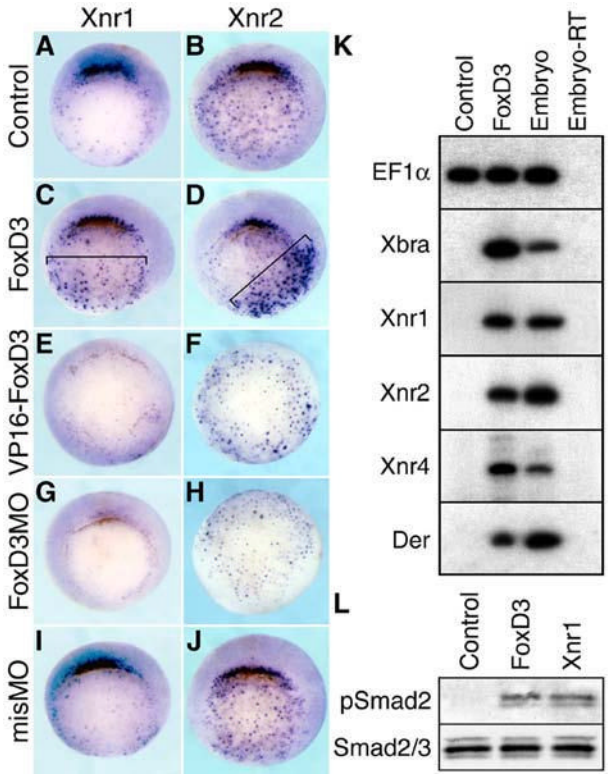

Figure 7.

FoxD3 is necessary and sufficient for *Nodal* expression. At the early gastrula stage, *Xnr1* (**A**) and *Xnr2* (**B**) are expressed in two distinct domains: strong expression in the dorsal marginal zone and punctate expression throughout the vegetal pole. In the experiment shown vegetal expression is more apparent for *Xnr2*. For FoxD3 gain-of function, 200 pg of FoxD3 RNA was injected into the marginal region of two blastomeres at the four-cell stage and the expression of *Xnr1* (**C**) and *Xnr2* (**D**) was examined by in situ hybridization at the early gastrula stage (stage 10.25). Ectopic expression of *Xnr1* and *Xnr2* is indicated with brackets. For FoxD3 loss-of-function, 0.5 ng of VP16-FoxD3 (**E,F**) or 25 ng of FoxD3MO (**G,H**) was injected into each blastomere at the four-cell stage and the expression of *Xnr1* and *Xnr2* was examined. As a negative control, 25 ng of mismatch MO (**I,J**) was injected. The results shown are representative of three independent experiments (n=20-25 embryos per sample in each experiment). Vegetal views with dorsal side up are shown. (**K**) At the one-cell stage, the animal pole was injected with FoxD3 RNA (300 pg) and animal explants prepared at the blastula stage (stage 9) were analyzed by RT-PCR at the early gastrula stage (stage 10.25) for the expression of *Brachyury* (Xbra), *Xnr1*, *Xnr2*, *Xnr4* and *Derriere* (Der). PCR controls are as described in Fig. 2. (**L**) Lysates of FoxD3- or Xnr1-expressing animal explants were examined for the presence of phospho-Smad2 protein by western blotting with a phospho-specific anti-Smad2 antibody. Stripped blots were analyzed for total Smad2/3 proteins as a loading control.

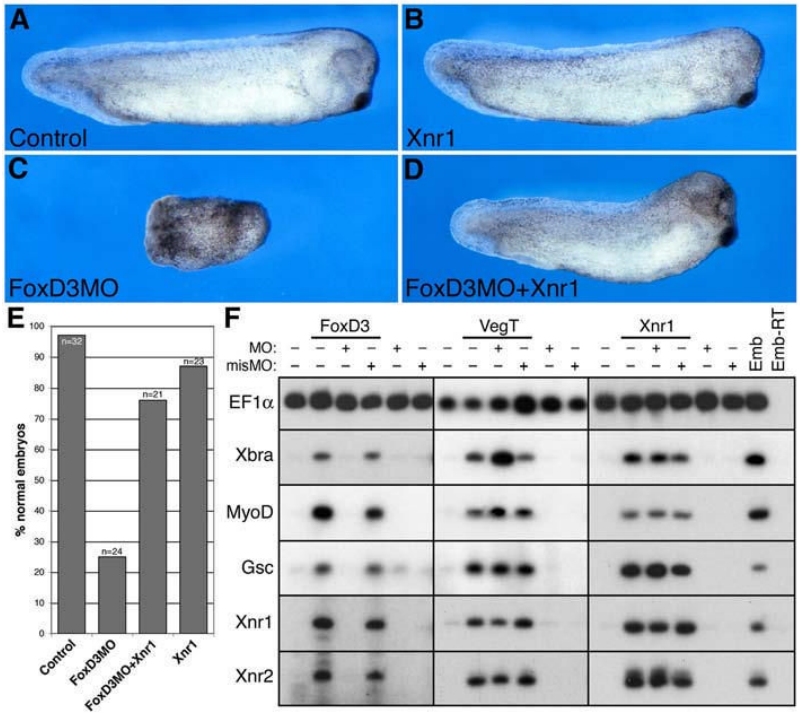

Figure 8.

FoxD3 acts upstream of Nodal in axis formation and mesoderm induction. At the four-cell stage, both dorsal blastomeres were injected with FoxD3MO (25 ng) alone (**C**), or in combination with 10 pg of *Xnr1* RNA (**D**). At the dose used, injection of Xnr1 alone (**B**) did not perturb axis formation in most embryos. (**E**) Quantification of a representative experiment. (**F**) To assess the dependence of Xnr1 and VegT activity on FoxD3, the animal pole was injected at the one-cell stage embryo with VegT (500 pg) or Xnr1 (100 pg) alone, or in combination with FoxD3MO or mismatch MO (50 ng). Animal explants were analyzed by RT-PCR at the gastrula stage (stage 11) for the expression of *Brachyury* (Xbra), *MyoD*, *Goosecoid* (Gsc), *Xnr1* and *Xnr2*. As controls, the oligonucleotides were injected alone or in combination with FoxD3 RNA (300 pg). PCR controls are as described in Fig. 2.

Table 1

FoxD3 rescue of axis formation in embryos injected with VP16-FoxD3 or FoxD3MO.

	N	Normal n (%)	Axial Defects n (%)
Uninjected	85	83 (98)	2 (2)
VP16-FoxD3	44	12 (27)	32 (73)
VP16-FoxD3+FoxD3	61	53 (87)	8 (13)
FoxD3MO	38	8 (21)	30 (79)
FoxD3MO+FoxD3(−utr)	54	49 (91)	5 (9)
FoxD3MO+FoxD3(+utr)	49	16 (33)	33 (67)
Mismatch MO	42	41 (98)	1 (2)
FoxD3	50	46 (92)	4 (8)

To determine the specificity of VP16-FoxD3 and FoxD3MO, *FoxD3* RNA was coinjected with VP16-FoxD3 or FoxD3MO to rescue axis formation. At the 4-cell stage, both dorsal blastomeres were injected with VP16-FoxD3 (0.5 ng) or FoxD3MO (25 ng) alone, or in combination with *FoxD3* RNA (25 pg), and axis formation was assessed at the tadpole stage (stage 35). As controls, both dorsal blastomeres were injected with mismatch MO (25 ng) or *FoxD3* RNA. Embryos in the "Axial Defects" class lacked head structures (eyes or cement gland absent) and had greatly reduced trunk and tail, while embryos in the "Normal" class had near normal head (eyes and cement gland present), trunk and tail structures. See Supplementary Materials (Fig. S4) for representative examples of phenotypic classes. N, total number of embryos; n, number of embryos in phenotypic class; %, percentage of embryos in phenotypic class.