FoxD3 regulation of Nodal in the Spemann organizer is essential for *Xenopus* dorsal mesoderm development

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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.

KEY WORDS: *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 by 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 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 days postcoitum (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 (see Fig. S1 in the supplementary material) (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 by 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

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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 co-expression 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 (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 (Kessler, 1997), 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 (see Fig. S2 in the supplementary material). 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 five positions (5′-ACAcGGTgATTCaAGTTACcCTgC-3′).

In situ hybridization, immunocytochemistry and histology
For whole-mount in situ hybridization, embryos were fixed and hybridized with antisense, digoxigenin-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 digoxigenin-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 previously 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). Radiolabeled 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 (see Fig. S1 in the supplementary material) (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 explant 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* mRNA was injected into a single ventral blastomere at the four-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 (see Fig. S3 in the supplementary material).

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 TGFβ-related proteins, which induce dorsal mesoderm formation, and Wnt activation of the βcatenin pathway, 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

Fig. 1. Ectopic axis induction by FoxD3. (**A**) Control. At the four-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).

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). Whereas 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, whereas 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 *αT4-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 TGFβ-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, whereas 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, whereas 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
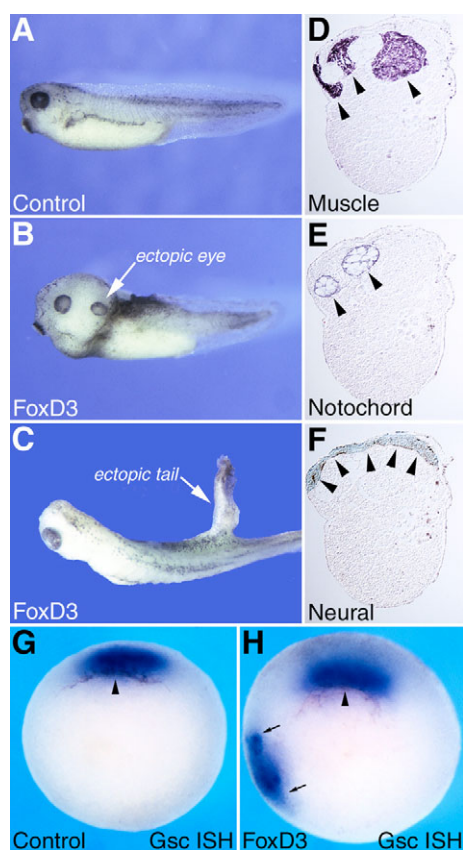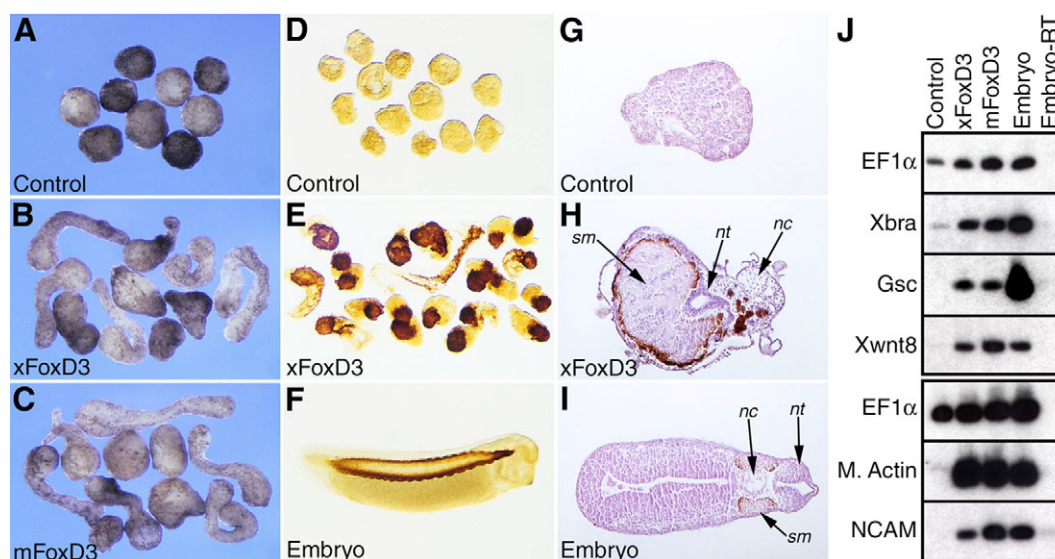
Fig. 2. Mesoderm induction by FoxD3. At the one-cell stage, embryos were injected in the animal pole with 200 pg of *Xenopus FoxD3* (xFoxD3) or mouse *FoxD3* (mFoxD3), explants were prepared at the late blastula stage (stage 9), and explants were analyzed for morphogenesis, tissue differentiation and 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).

transcriptional assay, FoxD3 repressed basal transcription of a luciferase reporter ~15-fold in animal explants at the gastrula stage (Yaklichkin et al., 2006). 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 co-expressed in animal explants and the induction of mesodermal markers was examined. Whereas FoxD3 induced *Muscle Actin* and *Collagen Type II*, this response was fully inhibited by co-expression 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 (see Fig. S2 in the supplementary material). 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, whereas a *FoxD3* RNA lacking the 5′UTR target sequence (FoxD3-utr) was translated
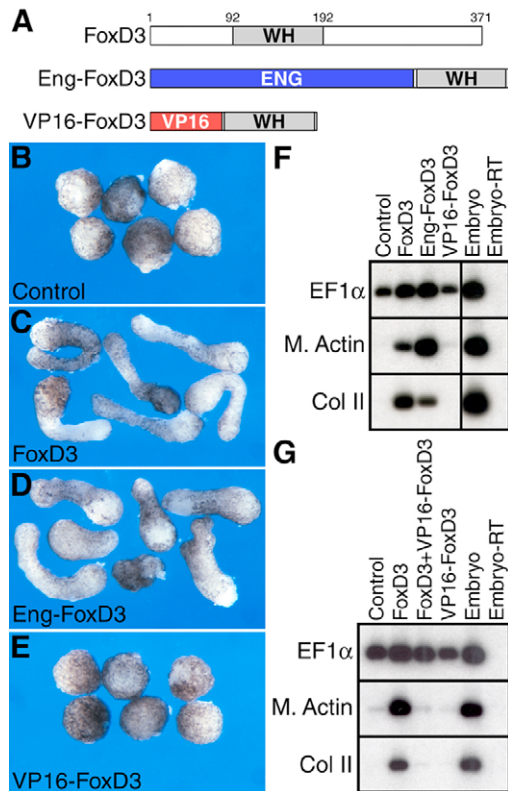
Fig. 3. Functional analysis of FoxD3 fusion proteins. (**A**) Schematic of the structure of FoxD3 and the FoxD3 fusion proteins. FoxD3 contains a conserved winged helix (WH) DNA-binding domain (residues 92-192). The repressor fusion protein (Eng-FoxD3) contains the repressor domain of *Drosophila* Engrailed (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, whereas VP16-FoxD3 did not. (**G**) Co-expression of VP16-FoxD3 and FoxD3 blocked induction of mesodermal genes by FoxD3. PCR controls are as described in Fig. 2.

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 ~tenfold 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

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 co-injected 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, whereas embryos in the 'normal' class had near-normal head (eyes and cement gland present), trunk and tail structures. See Fig. S4 in the supplementary material for representative examples of phenotypic classes. N, total number of embryos; n, number of embryos in phenotypic class; %, percentage of embryos in phenotypic class.

repressed by endogenous FoxD3, and that FoxD3MO will inhibit translation of endogenous FoxD3. At the four-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/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.
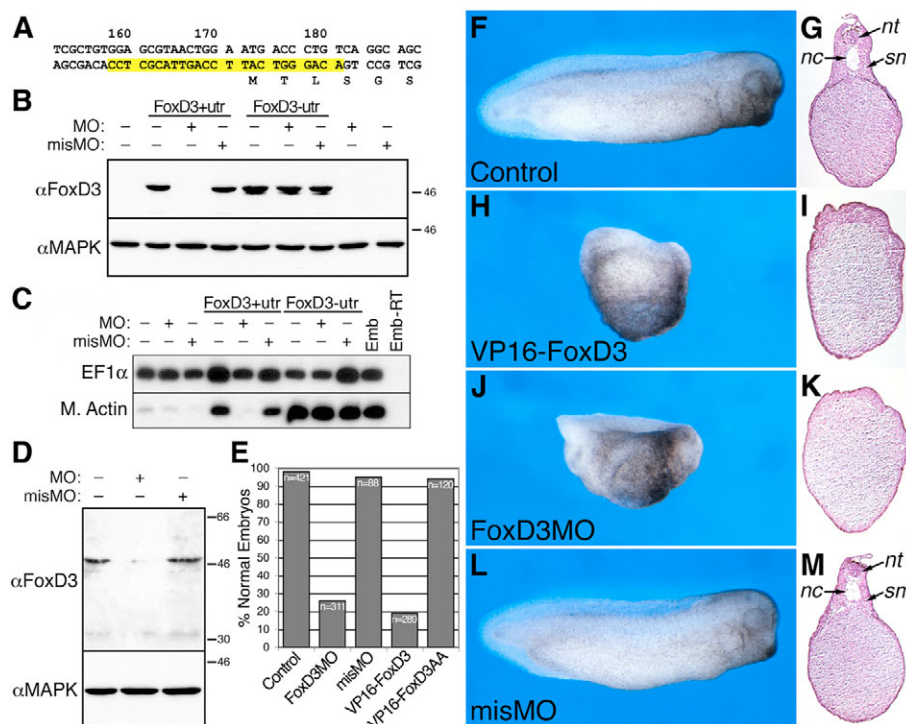
The inhibition of axis formation by VP16-FoxD3 and FoxD3MO is predicted to result from a specific block of endogenous FoxD3 function. To determine the specificity of FoxD3 inhibition, FoxD3 was co-injected with VP16-FoxD3 or FoxD3MO in an attempt to rescue axis formation (Table 1 and Fig. S4 in the supplementary material). Whereas the majority of VP16-FoxD3-injected embryos had severe axial defects (73%, n=44), only a minority displayed defects with FoxD3 co-injection (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 four-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), whereas 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 *TGFβ* 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).
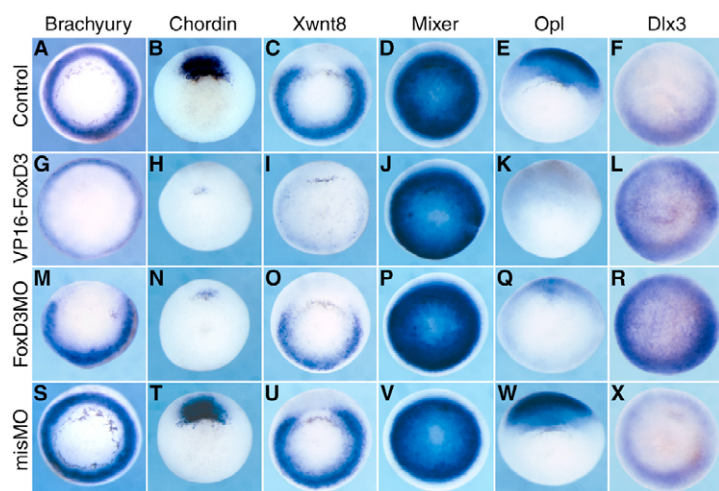

Fig. 4. FoxD3 function is required for axis formation. (**A**) The sequence of *FoxD3* flanking the initiator methionine with the sequence of the morpholino antisense oligonucleotide (181-158) highlighted in yellow. (**B**) Western analysis of animal explants prepared from embryos injected with *FoxD3* 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 ~tenfold 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.

Fig. 5. Mesodermal gene expression is dependent on FoxD3 function. (**A-F**) Control. 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.

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 co-expressed 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). Co-expression 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* and *Xnr4* 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). At the gastrula stage, *FoxD3* is co-expressed 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 were examined at the gastrula stage by
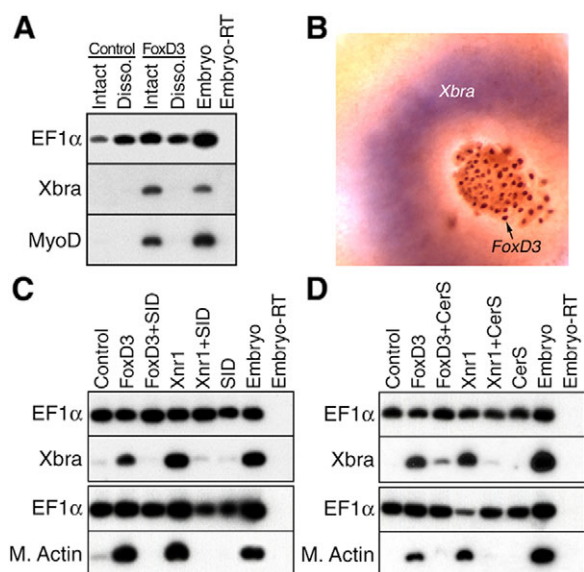

Fig. 6. Mesoderm induction by FoxD3 is non-cell-autonomous and dependent on the Nodal pathway. (**A**) At the one-cell stage the animal pole was injected with 100 pg of FoxD3 RNA and animal explants prepared at the early blastula (stage 7) were cultured intact or dissociated into individual cells in the absence of calcium (Disso.). 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.

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-

Fig. 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.

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 *TGFβ* family member co-expressed 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 four-cell stage, both dorsal blastomeres were injected with FoxD3MO alone, or in combination with *Xnr1* RNA. Whereas most embryos were affected by injection of FoxD3MO alone (75%, *n*=24), co-injection 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
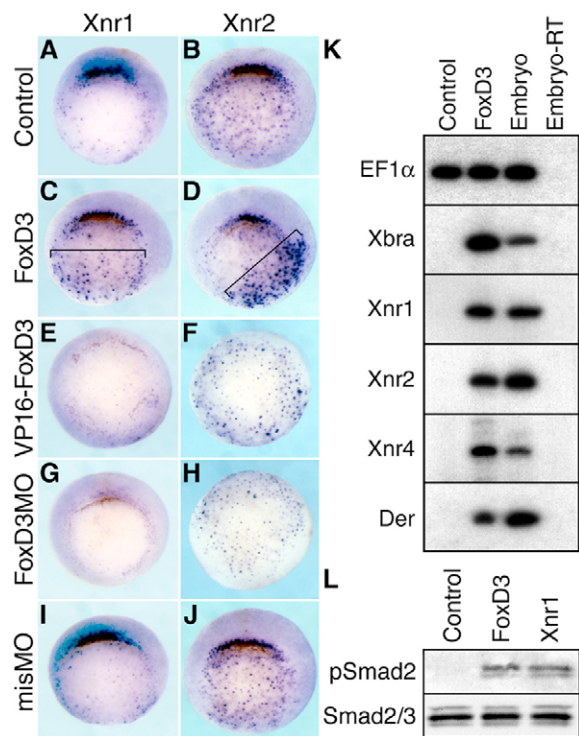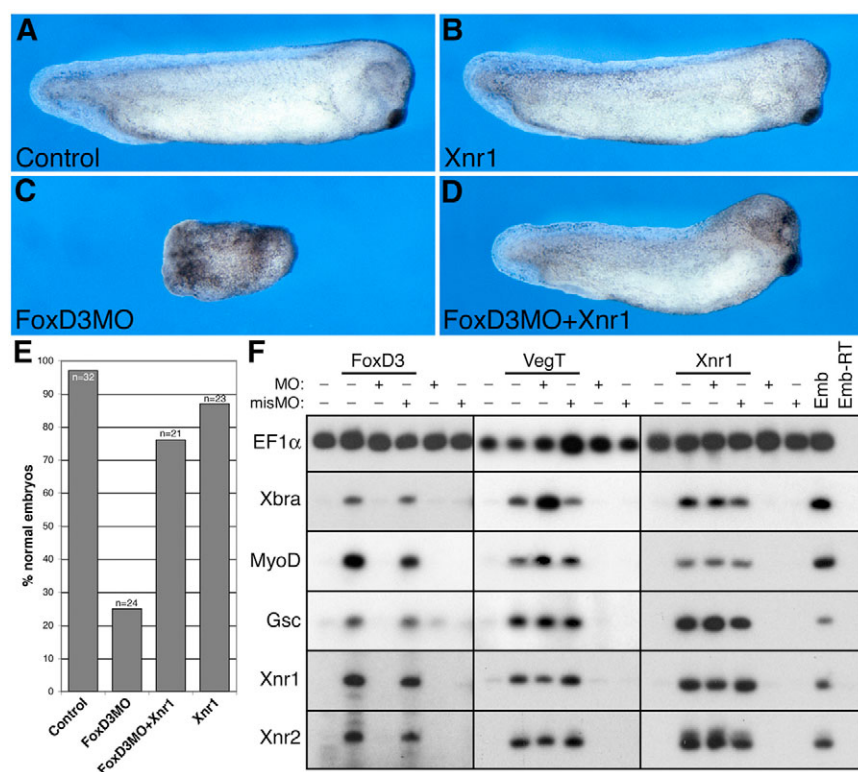
Fig. 8. FoxD3 acts upstream of Nodal in axis formation and mesoderm induction.
(**A**) Control. At the four-cell stage, both dorsal blastomeres were injected with FoxD3MO (25 ng) alone (**C**), or in combination with 10 pg of *Xnr1* RNA (**D**). At the dose used, injection of Xnr1 alone (**B**) did not 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.

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 co-expressed 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 βcatenin (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 co-repressors 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, as 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), whereas Dapper2, Smad7, Ectodermin and PIASy act intracellularly by stimulating receptor turnover or inhibiting Smad function (Nakao et al., 1997; Casellas and Brivanlou, 1998; Daniels et al., 2004; Zhang et al., 2004b; Dupont et al., 2005). The nuclear factors Drap1, Sox3, Xema and Zic2 inhibit the expression of *Nodal*-related genes or the transcriptional response to Nodal signals (Iratni et al., 2002; Zhang 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 thus 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. Although 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 co-expression 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 (Yaklichkin et al., 2006), 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 extra-embryonic ectoderm. *Foxd3* null embryos die at 6.5 dpc with a loss of epiblast cells and an expansion of extra-embryonic 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 is 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 ES 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
Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/133/24/4827/DC1

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