Xema, a *foxi*-class gene expressed in the gastrula stage *Xenopus* ectoderm, is required for the suppression of mesendoderm

Crystal Suri[1,2], Tomomi Haremaki[2] and Daniel C. Weinstein[2,*]

[1]Graduate training program in Mechanisms of Disease and Therapy, Mount Sinai School of Medicine, New York, NY 10029, USA
[2]Department of Pharmacology and Biological Chemistry, Mount Sinai School of Medicine, New York, NY 10029, USA
*Author for correspondence (e-mail: daniel.weinstein@mssm.edu)

Summary

The molecular basis of vertebrate germ layer formation has been the focus of intense scrutiny for decades, and the inductive interactions underlying this process are well defined. Only recently, however, have studies demonstrated that the regulated inhibition of ectopic germ layer formation is also crucial for patterning the early vertebrate embryo. We report here the characterization of Xema (*Xenopus* Ectodermally-expressed Mesendoderm Antagonist), a novel member of the Foxi-subclass of winged-helix transcription factors that is involved in the suppression of ectopic germ layer formation in the frog, *Xenopus laevis*. *Xema* transcripts are restricted to the animal pole ectoderm during early *Xenopus* development. Ectopic expression of *Xema* RNA inhibits mesoderm induction, both by growth factors and in the marginal zone, in vivo. Conversely, introduction of antisense morpholino oligonucleotides directed against the *Xema* transcript stimulates the expression of a broad range of mesodermal and endodermal marker genes in the animal pole. Our studies demonstrate that Xema is both necessary and sufficient for the inhibition of ectopic mesendoderm in the cells of the presumptive ectoderm, and support a model in which Fox proteins function in part to restrict inappropriate germ layer development throughout the vertebrate embryo.

Key words: *Xenopus*, *Xema*, Mesendoderm, Mesoderm, Fox

Introduction

All tissues in an animal derive from the three primary germ layers: ectoderm, endoderm and mesoderm. Ectodermal derivatives constitute both the central nervous system and the epidermis, while endodermal derivatives give rise to a number of organs, including the lungs, liver, pancreas, thyroid gland and thymus, as well as to the entire gastrointestinal epithelium. The mesodermal germ layer plays a pivotal role in organizing the vertebrate body axes, and itself gives rise to the muscular, skeletal and circulatory systems. Much of our understanding of mesoderm formation has come from classical and molecular studies in the amphibian embryo. In the frog *Xenopus laevis*, mesoderm is an inducible cell fate: although vegetal pole explants form only endodermal derivatives and animal pole ('animal cap') explants form only ectodermal derivatives, signals from cells of the vegetal pole generate the additional formation of mesoderm in overlying animal caps when these explants are recombined (Slack et al., 1984). These observations are consistent with the amphibian fate map: mesoderm forms in the so-called 'marginal zone', at the border between the animal and vegetal poles (Dale and Slack, 1987).

Two classes of secreted molecules have been found to possess mesoderm-inducing activity. Addition of Fibroblast Growth Factor (Fgf) or members of the Activin/Nodal-related class of the Transforming Growth Factor β (Tgfβ) ligand family will induce mesoderm in explants of competent ectoderm, whereas inhibition of the signaling cascades downstream of these factors blocks mesoderm formation in vivo (Harland and Gerhart, 1997; Slack, 1994). Although considerably less attention has been focused on the signals governing endoderm formation, recent studies have demonstrated that this germ layer is also dependent on Tgfβ signaling, as well as on a cascade of transcription factors that act cell autonomously to ensure commitment to an endodermal fate (Shivdasani, 2002). The recognition that similar mechanisms underlie the formation of both mesoderm and endoderm are reflected in the notion of a 'mesendodermal' field in the early embryo, containing both mesodermal and endodermal precursors, from which the distinct germ layers emerge only during gastrulation. A second, distinct domain thought to contain only mesodermal precursors has also been described (Rodaway and Patient, 2001). In this report, we will use the term 'mesendoderm' somewhat more loosely, to describe all early mesodermal and/or endodermal cells. Although the mechanisms regulating early patterning of the vertebrate ectoderm have been the subject of considerable debate, it has, until recently, been generally assumed that the development of the presumptive ectoderm is governed in large part by the absence of mesoderm- or endoderm-promoting cues.

Although growth factor-mediated induction clearly drives

mesoderm formation in the vertebrate embryo, recent studies in the mouse and frog suggest that germ layer suppression is also crucial in establishing the early vertebrate body plan. For example, the Tgfβ ligand Nodal plays an essential role during mesoderm and endoderm formation in the mouse, as nodal mutant mice lack a primitive streak, a posterior structure from which embryonic and extra-embryonic mesoderm, and embryonic endoderm are derived (Conlon et al., 1994; Whitman, 2001; Zhou et al., 1993). Surprisingly, prior to gastrulation, Nodal is transiently expressed throughout the early epiblast (Whitman, 2001). Recent studies suggest that the anterior visceral endoderm (AVE), an extraembryonic tissue overlying the anterior epiblast, is a crucial source of Nodal antagonism (Beddington and Robertson, 1999; Perea-Gomez et al., 2002); the AVE expresses at least two secreted Nodal antagonists, Cerberus-like (Cer1) and a Tgfβ superfamily molecule, Lefty1 (Perea-Gomez et al., 2001). A subset of compound $Cer1^{-/-};Lefty1^{-/-}$ mutant embryos develop ectopic primitive streaks (Perea-Gomez et al., 2002); this phenotype is partially rescued in mice containing a single copy of Nodal, suggesting that Cer1 and Lefty1, secreted by the AVE, function to restrict ectopic streak formation by inhibiting Nodal activity anteriorly. Recent evidence points to Nodal antagonism at several additional levels during early development: both the transcriptional co-repressor DRAP1, and Dpr2, which promote the lysosomal degradation of Nodal receptors, have been shown to limit Nodal activity in the vertebrate embryo (Iratni et al., 2002; Zhang et al., 2004).

Several recent studies have suggested that inhibition of ectopic germ layer formation is also crucial during early development of the frog Xenopus laevis. For example, an analysis of the Xenopus brachyury promoter found that the restricted expression of this gene in the cells of the mesoderm is in part the result of specific repression in both endoderm and ectoderm; along with one or more as yet undefined homeodomain proteins, the δEF1 repressor family Smad-interacting protein-1 (Sip1) appears to be required for this repression (Lerchner et al., 2000). Recently, Wardle and Smith reported the presence of a significant number of cells found throughout the early gastrula-stage Xenopus embryo that express mesendodermal markers inappropriate to their location and, on occasion, do not correspond to any single regional fate (Wardle and Smith, 2004); these 'rogue' cells are largely undetectable by late gastrula stages, suggesting that they are either converted to the fate of their neighbors, or eliminated via as yet uncharacterized mechanisms. Finally, the Forkhead box (Fox) DNA-binding protein Hepatocyte Nuclear Factor 3β (Hnf3β)/Foxa2, expressed throughout the Xenopus deep endoderm at early gastrula stages, also inhibits inappropriate germ layer development in the frog: misexpression of Hnf3β in the marginal zone leads to a loss of mesoderm and dorsal mesendoderm, whereas suppression of Hnf3β target genes leads to ectopic axis formation in the endoderm (Suri et al., 2004). Consistently, gene targeting studies have implicated a requirement for murine Hnf3β in mesoderm suppression: targeted deletion of both Hnf3β and the LIM homeodomain protein Lim1 in the visceral endoderm leads to the production of ectopic ventral mesoderm in the epiblast, a phenotype not observed following the deletion of either gene alone (Ang and Rossant, 1994; Dufort et al., 1998; Perea-Gomez et al., 1999; Weinstein et al., 1994). Thus, the expression of Hnf3β in the

Xenopus deep endoderm and the mammalian extra-embryonic endoderm appears to be involved in the suppression of ectopic mesendoderm in, respectively, both lower and higher vertebrates. The molecular basis for this requirement, however, remains largely unknown.

We report here that a Fox-mediated, mesendoderm-suppressing mechanism is also active within the Xenopus ectoderm, and is required for normal development. We describe the characterization of Xema, a Fox protein expressed exclusively in the ectoderm during early Xenopus development. Misexpression of Xema blocks mesendoderm induction in vivo and by inducing agents, while Xema knockdown promotes mesendoderm development in gastrula stage animal cap explants, demonstrating a requirement for Xema in the inhibition of ectopic germ layer formation in the animal pole. Taken together with studies on Hnf3β from our laboratory and others, this body of research supports a model in which Fox proteins are fundamental mediators of the widespread suppression of ectopic germ layer development during early vertebrate embryogenesis.

Materials and methods

RNA preparation, explant dissection and cell culture
RNA was synthesized in vitro in the presence of cap analog using the mMessage mMachine kit (Ambion). Microinjection, explant dissection and cell culture were performed as described (Hemmati-Brivanlou and Melton, 1994; Wilson and Hemmati-Brivanlou, 1995).

RT-PCR
RT-PCR was performed as described (Wilson and Hemmati-Brivanlou, 1995). Primers designed specifically for this study were as follows: Xema-U, 5′-AGTAGGTCAGTTCCACTTGG; Xema-D, 5′-AAGGACTTTGTCGTGACTGC. All other primer sequences were as described previously (Suri et al., 2004).

Subtractive hybridization screen
PCR-based differential screens were performed to identify genes with expression enriched in or limited to the gastrula-stage ectoderm, relative to levels found in other regions of the embryo or after treatment with various mesoderm-inducing reagents. In the screen from which Xema was isolated, cDNA was generated from 1 µg of mRNA isolated from uninjected animal caps and animal caps injected with EnR-Hnf3β RNA; expression of this construct generates ectopic mesoderm in both endoderm and ectoderm (Suri et al., 2004) (data not shown). cDNA pools were hybridized and differentially expressed genes were selected using a PCR-based subtraction method (Clontech PCR-Select cDNA Subtraction kit), both in the laboratory and using the 'Custom PCR-Select Subtraction Analysis' service (Clontech). Clones whose expression was found to be downregulated in induced explants were selected, amplified and sequenced. Primers were generated and RT-PCR was performed to confirm differential expression using cDNA derived from independent experimental samples.

Whole-mount in situ hybridization, immunohistochemistry and β-gal staining
Protocols for whole-mount in situ hybridization were derived from Harland (Harland, 1991) with the following changes: (1) RNase steps were eliminated; and (2) BM purple AP substrate (Boehringer Mannheim) replaced BCIP/NBT. The antisense Xema and Xbra probes were synthesized in the presence of digoxigenin-11-UTP (Boehringer Mannheim). Whole-mount β-gal detection was performed as described (Smith and Harland, 1992). Whole-mount

antibody staining was performed as described (Hemmati-Brivanlou and Melton, 1994). The 12/101 antibody (ascites, Developmental Studies Hybridoma Bank) was used at a 1:1 dilution. Secondary antibody was a donkey anti-mouse IgG coupled to horseradish peroxidase (Jackson Laboratories), and was used at a 1:1000 dilution. Color reactions were performed using the Vector SG and DAB kits (Vector Laboratories).

Preparation of *Xema* fusion proteins

Xema fusion constructs were generated by PCR. For EnR-Xema, residues 1-298 of the *Drosophila* Engrailed repressor (Kessler, 1997) were fused upstream of full-length Xema. For VP-Xema, residues 410-490 of the VP16 activator (Kessler, 1997) were fused upstream of full-length Xema.

Morpholinos

Morpholino oligonucleotides (Gene Tools) were heated for five minutes at 65°C then quenched on ice, prior to injection at the two- or four-cell stage. Sequences were as follows: Xema MO-1, GT-GCTTGTGGATCAAATGCACTCAT; Xema MO-2, AGGTCA-CAAATACACCTGTACTAGC; control morpholino (mismatch-1/MM-1), GTcCTTGTaATgAAATcCACTgAT

Results

Identification of the *foxi* gene *Xema*

In an attempt to identify factors involved in germ layer suppression, we performed a PCR-based differential screen to select for genes that are downregulated in gastrula stage ectoderm following mesoderm-inducing treatments. cDNA clone A07 was one of several isolates whose expression was strongly suppressed by the Tgfβ ligand Activin (Fig. 1A). Database searches revealed that the corresponding full-length transcript encodes a novel Fox transcription factor, of the Foxi class (Fig. 1B) (Kaestner et al., 2000). Although A07 shares the highest homology with murine Foxi1, a distinct *Xenopus* gene with lower overall homology has already been designated Foxi1 (Lef et al., 1994). Among lower vertebrate Foxi genes, A07 is most similar to zebrafish Foxi3; furthermore, the DNA-binding domain of A07 appears identical to a partial *Xenopus* sequence described elsewhere as "Foxi3" (Solomon et al., 2003). As we cannot currently provide the standard nomenclature for this gene beyond an assignation to the Foxi subclass, we refer to it, for reasons that will become clear, as *Xema* (*Xenopus* Ectodermally-expressed Mesendoderm Antagonist, pronounced 'zee-ma').

In order to determine the temporal range of *Xema* expression during early development, we performed reverse-transcription polymerase chain reactions (RT-PCR) on RNA harvested from embryos at various embryonic stages. *Xema* is not expressed maternally, as transcripts are present only after the initiation of zygotic transcription at stage 8.5 (Fig. 2A). Expression is highest at early gastrula stages (stage 10) and is maintained throughout neurula, tailbud and early tadpole stages. To analyze the spatial distribution of *Xema* transcripts, we performed whole-mount in situ hybridization studies. *Xema* transcripts were observed in the cells of the animal pole at late blastula and gastrula stages (Fig. 2B and data not shown). *Xema* transcripts were not detected in the marginal zone during early (stage 10+) or mid (stage 11) gastrula stages; *Xbra* expression, restricted to the gastrula marginal zone, is shown for comparison (Fig. 2B) (Smith et al., 1991). *Xema* expression

is restricted to the ventral ectoderm during early neurula stages; this epidermal expression persists through tailbud stages (Fig. 2B and data not shown). In order to confirm the regional distribution of *Xema* transcripts, we performed RT-PCR analysis on early gastrula stage explants. These assays support the in situ analysis: *Xema* is expressed in the animal pole (AP) ectoderm, and is excluded from the ventral and dorsal marginal zone (VMZ and DMZ), as well as the vegetal pole (VP) (Fig. 2C). Thus, *Xema* is expressed zygotically in the ectoderm during early *Xenopus* development, and is excluded from regions of the embryo that contribute to mesodermal and endodermal lineages.

Ectopic Xema suppresses mesendoderm formation

We have previously demonstrated that the Fox gene Hnf3β is expressed throughout the deep endoderm during *Xenopus* gastrulation, and is involved in suppressing ectopic mesoderm and dorsal mesendoderm in this region (Suri et al., 2004). The gastrula-stage expression of *Xema* suggested that this Fox gene might be involved in the suppression of ectopic germ layer formation in the presumptive ectoderm. Dorsal marginal zone expression of *Xema* RNA gives rise to embryos, the majority of which lack heads and show little discernable polarity (69%;
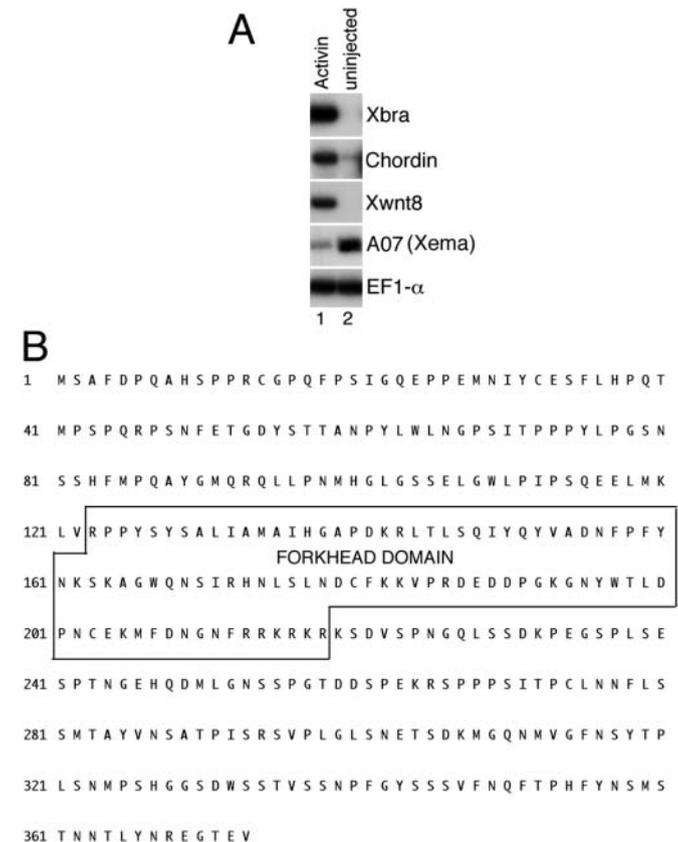

Fig. 1. Identification of the *Xenopus* Foxi-class gene *Xema* (GenBank accession number DQ026065). (A) Activin inhibits A07 (Xema) expression. RT-PCR analysis of animal cap explants dissected at late blastula stages and cultured until midgastrula stages. Activin (0.5 ng/ml) was added to stage 9 animal caps, as listed. (B) Putative *Xema* amino acid sequence. The forkhead box (Fox) DNA-binding domain is indicated.
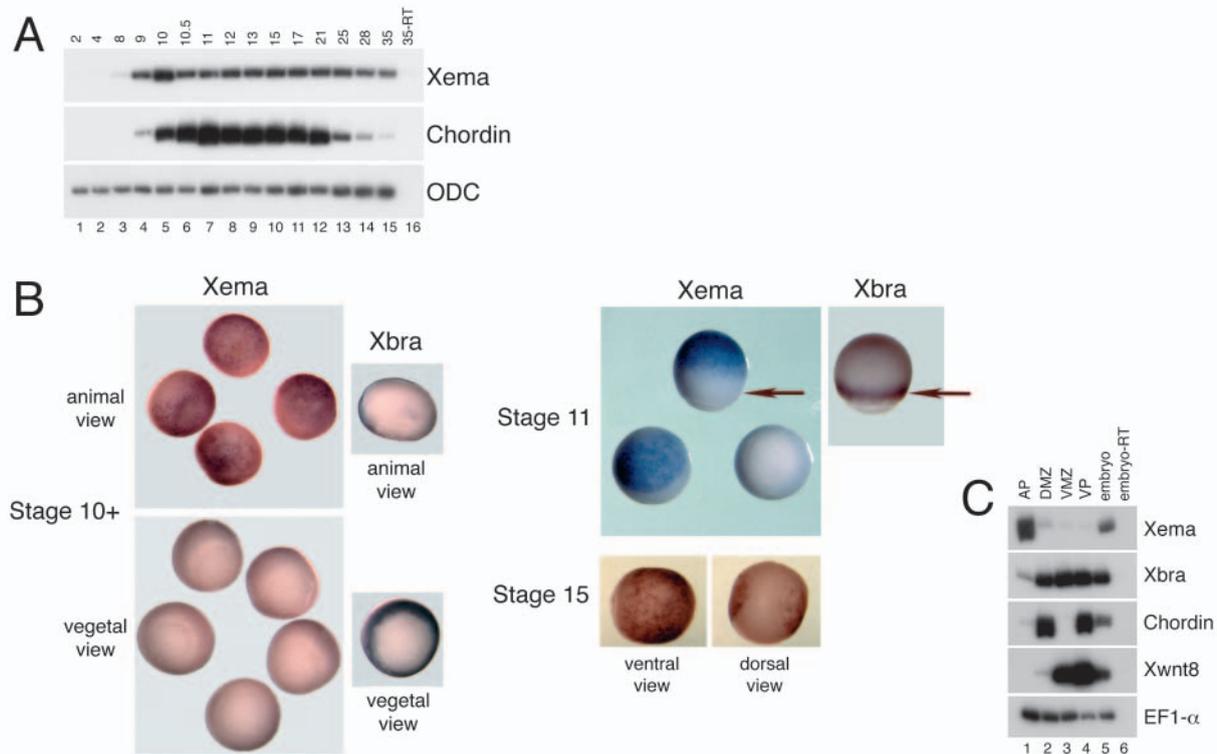
Fig. 2. Expression of *Xema* during early development. (A) RT-PCR analysis of *Xema* temporal expression. The '–RT' lane contains all reagents except for reverse transcriptase and was used as a negative control. Ornithine decarboxylase (ODC) was used as a loading control (Bassez et al., 1990). *Chordin* expression is initiated zygotically and was used as a staging control. (B) Whole-mount in situ hybridization of early gastrula (stage 10+), midgastrula (stage 11), and early neurula (stage 15) stage embryos, using antisense *Xema* and *Xenopus brachyury* (*Xbra*) probes. *Xema* expression is seen as a blue stain throughout the animal pole of gastrula-stage albino embryos; *Xema* expression is excluded from the marginal zone (denoted by arrows at stage 11) and vegetal pole. Expression of the panmesodermal marker *Xbra* is found only in the marginal zone of gastrula stage embryos and was used as a control. *Xema* is expressed in the vental ectoderm (epidermis) of early neurula stage embryos (ventral view); expression is excluded from the neural plate (dorsal view). (C) RT-PCR analysis of *Xema* expression in early gastrula stage explants. EF1-α was used as a loading control (Krieg et al., 1989). *Xbra* is a panmesodermal marker at this stage (Smith et al., 1991), *chordin* is a dorsal endomesodermal marker (Sasai et al., 1994), and *Xwnt8* is a ventrolateral marker (Christian et al., 1991; Smith and Harland, 1991).

n=35); ventral injection of *Xema* RNA results in variable and more modest defects in trunk and tail development (31% with clear abnormalities; *n*=29) (Fig. 3A). Whole-mount immunohistochemistry with the somite-specific antibody 12/101 demonstrated that formation of the somites, a paraxial mesoderm derivative, is dramatically inhibited by dorsal injection of *Xema* RNA (Fig. 3B) (Kintner and Brockes, 1984). Consistently, Xema misexpression in dorsal marginal zone (DMZ) explants leads to a reduction of *Xbra* expression, as well as a dramatic decrease in the expression of dorsal endomesodermal markers, including *chordin* and *goosecoid* (Fig. 3C, compare lanes 1 and 2) (Cho et al., 1991; Sasai et al., 1994). We found that Xema expression in dorsal cells often leads to a reduction in or absence of dorsal lip invagination (see Fig. 3D, and data not shown). Introduction of *Xema* RNA into ventral cells leads to a strong reduction in the expression of both *Xbra* and the ventrolateral marker *Xwnt8* (Fig. 3C; compare lanes 3 and 4) (Smith and Harland, 1991); VMZ inhibition of *Xbra* is often more dramatic than that observed in the DMZ (Fig. 3C and data not shown). Marginal zone expression of *epidermal keratin* is upregulated in dorsal but not ventral explants, suggesting that Xema misexpression may promote ectodermal fates in some contexts, in addition to

inhibiting mesendodermal ones (Fig. 3C). Consistent with our explant data, *Xbra* expression is also blocked by *Xema* RNA injection in both the dorsal and ventral marginal zones in intact embryos, as demonstrated by whole-mount in situ hybridization analysis (Fig. 3D).

We next examined Xema function in animal pole ectodermal explants in the presence or absence of growth factors. Animal caps from uninjected or *Xema*-injected embryos were first treated with the Tgfβ ligand Activin. At the doses used for these studies, Activin induces the expression of a range of mesodermal and endodermal markers at gastrula stages (Fig. 3E; compare lanes 1 and 2), all of which are inhibited by the injection of *Xema* RNA (Fig. 3E; compare lanes 2 and 3), including *Xbra*, *Xwnt8*, *chordin*, the panendodermal marker *Sox17β*, and the anterior endodermal markers *cerberus* and *Xhex* (Bouwmeester et al., 1996; Hudson et al., 1997; Jones et al., 1999; Newman et al., 1997). Injection of *Xema* RNA does not rescue the modest reduction of *epidermal keratin* expression seen in Activin-treated caps (Fig. 3E; compare lanes 1, 2, and 3). Finally, ectopic Xema also inhibits ventral mesoderm induced by Fgf (Fig. 3F). These data demonstrate that Xema can inhibit mesoderm and endoderm formation in a variety of assays and, coupled with the expression data
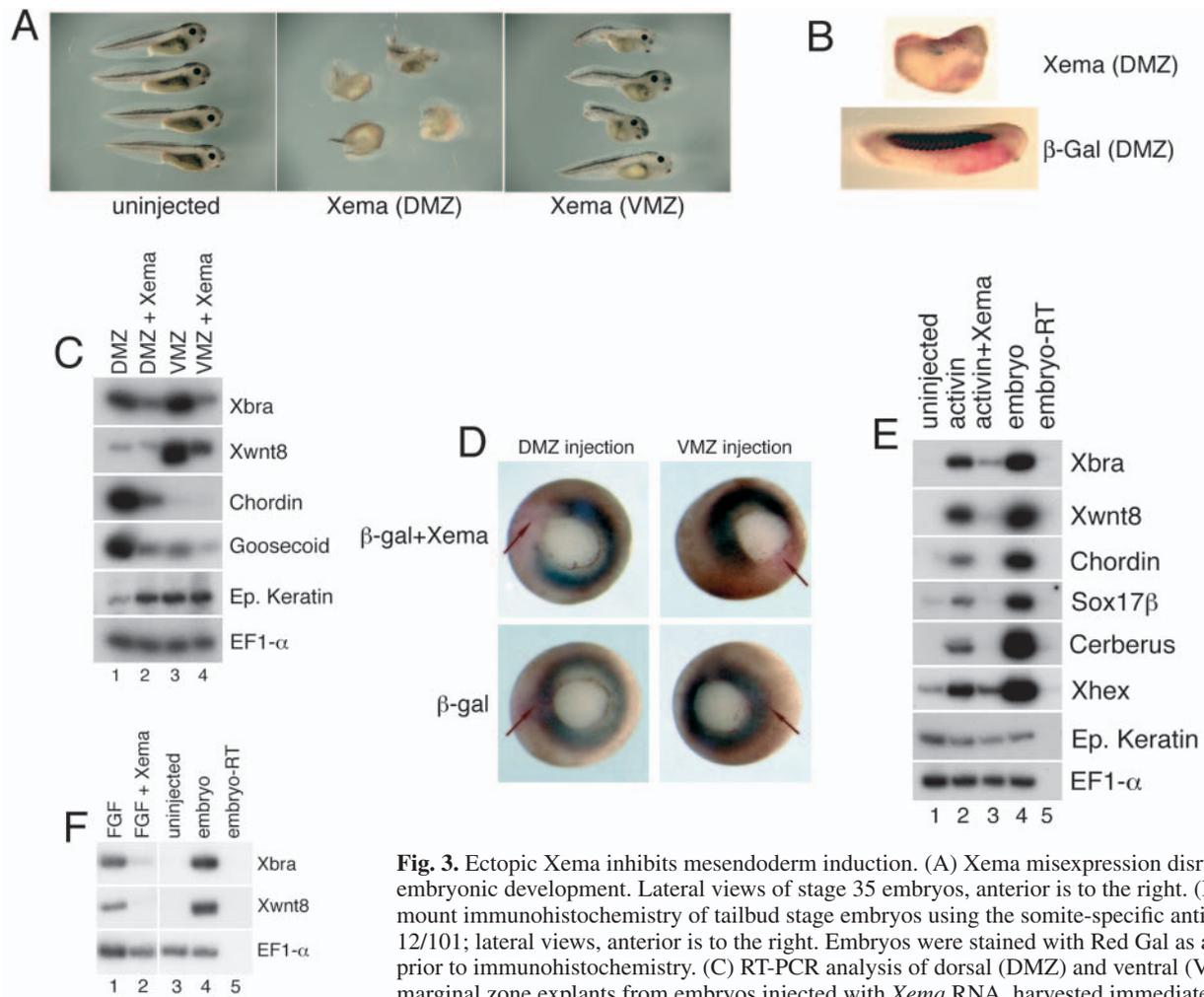
Fig. 3. Ectopic Xema inhibits mesendoderm induction. (A) Xema misexpression disrupts embryonic development. Lateral views of stage 35 embryos, anterior is to the right. (B) Whole-mount immunohistochemistry of tailbud stage embryos using the somite-specific antibody 12/101; lateral views, anterior is to the right. Embryos were stained with Red Gal as a substrate prior to immunohistochemistry. (C) RT-PCR analysis of dorsal (DMZ) and ventral (VMZ) marginal zone explants from embryos injected with *Xema* RNA, harvested immediately after dissection at early gastrula stages. (D) Whole-mount in situ hybridization of midgastrula stage embryos, using an antisense *Xenopus brachyury* probe; vegetal pole views are shown. Embryos were stained with Red Gal as a substrate prior to in situ hybridization. The embryos shown at the top of the figure were co-injected with *lacZ* RNA and *Xema* RNA in two dorsal or ventral blastomeres, at the four-cell stage, as listed; note the presence of β-galactosidase activity (red, arrows) in the gap in *Xenopus brachyury* expression (blue). The embryos shown at the bottom of the figure were injected with *lacZ* RNA in the same regions, as indicated; note the overlap (arrows) between *Xenopus brachyury* expression and β-galactosidase activity. (E) Inhibition of Activin-mediated mesendoderm induction by Xema. RT-PCR analysis of animal cap explants dissected at late blastula stages and cultured until midgastrula stages. *Xema* RNA was injected into the animal pole region of both blastomeres at the two-cell stage. Activin (0.5 ng/ml) was added to stage 9 animal caps, as listed. (F) Inhibition of Fgf-mediated mesoderm induction by Xema. bFgf (10 ng/ml) was added to stage 9 animal caps, as listed. For all experiments in this figure, 1 ng of *Xema* RNA, and/or 100 pg *lacZ* RNA was injected, as listed.

described above, suggest that endogenous Xema may be involved in suppressing non-ectodermal fate in the animal pole.

Activation of Xema target genes phenocopies Xema misexpression

The *Fox* genes encode a large class of nuclear DNA-binding factors; although members of the Foxi-class generally appear to stimulate the transcription of target genes, Fox proteins include both transcriptional activators and repressors (Carlsson and Mahlapuu, 2002). To address the mechanism by which Xema inhibits mesendoderm formation, we sought to determine whether this protein functions as a transactivator or repressor during early development. Towards this end, we generated an activated Xema construct, VP-Xema, by fusing the full-length Xema cDNA to the VP16 activation domain (Kessler, 1997). Microinjection of *VP-Xema* RNA inhibits

mesoderm induction by Activin (Fig. 4A). These results mimic the effects seen following expression of wild-type Xema and, coupled with repressor data (see below), suggest that Xema functions as a transcriptional activator during early development.

Repression of Xema target genes induces mesoderm formation

We next sought to determine whether repression of Xema target genes promotes mesendoderm. For these studies, we constructed a *Drosophila* Engrailed repressor-*Xema* fusion protein, EnR-Xema (Kessler, 1997). Gastrula stage animal pole explants excised from embryos injected with *EnR-Xema* express both *Xbra* and *Xwnt8*; expression of the dorsal endomesodermal marker *chordin* is not stimulated by EnR-Xema (Fig. 4B). As expected, EnR-Xema does not inhibit
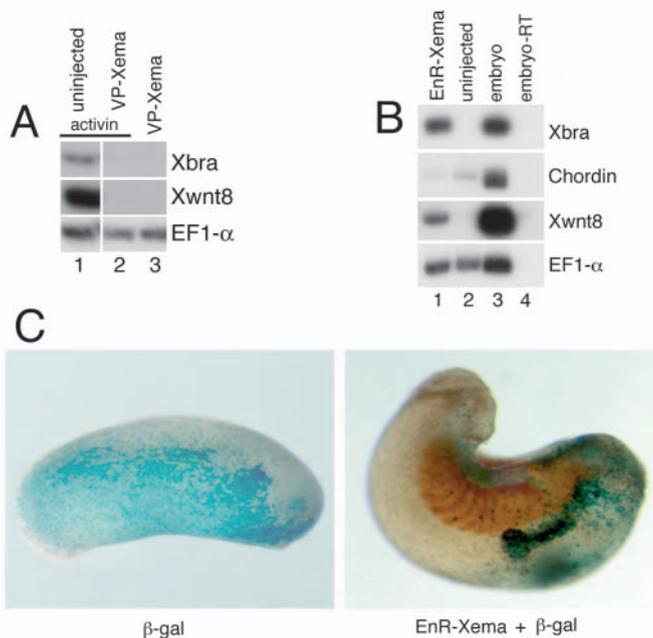
Fig. 4. Activity of Xema activator and repressor constructs. (A) VP-Xema-mediated inhibition of mesoderm formation. RT-PCR analysis of animal caps dissected at late blastula stages and cultured until midgastrula stages. *VP-Xema* RNA (1 ng) was injected at early cleavage stages, as indicated. Activin (0.5 ng/ml) was added to stage 9 animal caps, as indicated. (B) Expression of EnR-Xema induces mesoderm. RT-PCR analysis of animal caps dissected at late blastula stages and cultured until midgastrula stages. *EnR-Xema* RNA (1 ng) was injected at early cleavage stages, as indicated. (C) Expression of EnR-Xema induces ectopic structures. Whole-mount immunohistochemistry of tailbud stage embryos using the somite-specific antibody 12/101; lateral views, anterior is to the right. Embryos were stained with X-Gal as a substrate prior to immunohistochemistry. The embryo on the right was co-injected with 100 pg *lacZ* and 1 ng *EnR-Xema* RNA in the animal pole at the two-cell stage; note the absence of somite staining (red) in the secondary structure containing the β-galactosidase activity (blue). The embryo on the left was injected with 100pg *lacZ* RNA in the animal pole at the two-cell stage and was not probed with the somite-specific antibody.

mesoderm induction by Activin and, in fact, modestly synergizes with low levels of Activin (data not shown). These data suggest that the transcriptional repression of one or more genes by EnR-Xema is sufficient to stimulate mesodermal differentiation in the presumptive ectoderm.

Ectodermal EnR-Xema expression in intact embryos leads both to mild defects in head development and to the formation of ectopic lateral structures, often near the heads of affected embryos (62%; *n*=55) (Fig. 4C). Lineage tracing experiments demonstrated that the ectopic structures contain a high proportion of the microinjected cells (Fig. 4C, and data not shown). Although these secondary structures occasionally resemble ectopic tails, they do not usually contain dorsal mesoderm: a 'true' secondary axis was seen in only one embryo expressing EnR-Xema, and an antibody against a somite-specific epitope detected the presence of paraxial mesoderm in one additional embryo (data not shown). This phenotype is reminiscent of that seen following activation of

the fibroblast growth factor (Fgf) signaling cascade (Isaacs et al., 1994; Weinstein et al., 1998).

Xema knockdown induces mesoderm

We have shown that Xema misexpression in the marginal zone blocks mesendoderm formation, and that repression of Xema target genes results in ectopic mesoderm. These data, coupled with the localization of *Xema* transcripts in the early *Xenopus* ectoderm, suggest that this factor is normally involved in inhibiting ectopic germ layer development in the animal pole. Results with the EnR-Xema construct, however, may differ from loss-of-function studies in several crucial ways. First, because EnR-Xema is likely to function as a dominant-negative reagent, other related proteins may act redundantly with Xema during mesendodermal suppression in vivo. Second, and perhaps more crucially, EnR-Xema may repress the transcription of genes in the ectoderm to which Xema can bind but does not normally activate. To determine whether mesendodermal suppression by Xema is required for normal development, we attempted to inhibit Xema function using antisense morpholino oligonucleotides. We designed and purchased (from GeneTools) two morpholinos against Xema, MO-1 and MO-2, both of which effectively block the translation of *Xema* RNA in vitro (Fig. 5A; compare lane 1 with lanes 3 and 4). Co-expression of MO-1 and MO-2 leads to a more effective inhibition of Xema translation than is observed with either morpholino alone (Fig. 5A; compare lanes 3, 4 and 5). Expression of a control morpholino, identical to MO-1 with the exception of five base-pair mismatches (mismatch-1/MM-1), has no effect on Xema translation (Fig. 5A; compare lanes 1 and 2). Consistent with the in vitro data, we find that injection of either MO-1 or MO-2 inhibits the expression of a 3′-Myc-tagged Xema protein (Xema-Myc) in *Xenopus* embryos (Fig. 5B; compare lane 1 with lanes 3 and 4). Co-expression of MO-1 and MO-2 causes a greater block to expression than does injection of either morpholino alone and, as expected, Xema MM-1 does not inhibit the expression of Xema-Myc (Fig. 5B). These data suggest that Xema MO-1 and MO-2 act specifically to block translation of *Xema* RNA in vitro and in the context of the early embryo.

We next attempted to use the Xema morpholino oligonucleotides in 'knockdown' loss-of-function studies. For these experiments, the same doses of MO-1 and MO-2 were used as for the Xema-Myc assay shown in Fig. 5B. Injection of either Xema MO-1 (Fig. 5C) or MO-2 (see Fig. 5D, and data not shown) stimulates expression of *Xbra*, *Xwnt8*, *chordin* and the endodermal marker *Sox17β* in animal cap explants, and reduces the expression of the ventral ectodermal marker *epidermal keratin* (Jonas et al., 1985); crucially, injection of the control morpholino MM-1 has no effect on mesodermal or endodermal gene expression (Fig. 5C). Most strikingly, co-injection of MO-1 and MO-2 leads to a strong increase in the expression of a number of endodermal and mesodermal markers, including *Xbra*, *Xwnt8*, *chordin*, *Xnr3*, *goosecoid*, *Sox17β* and *Xhex*, none of which are expressed in control ectodermal explants (Fig. 5D). It has been difficult to observe the effects of Xema knockdown in intact embryos past the late blastula stage: doses required to generate mesendodermal marker expression in intact caps are lethal in gastrula stage embryos, with toxicity readily apparent by stage 10+; we have thus not been able to score for axial duplications or ectopic

expression of marker genes by in situ hybridization. Co-injection of 2-fold lower doses of the Xema morpholinos, however, does result in the formation of secondary lateral structures, resembling those observed following *EnR-Xema* RNA injection, in 52% of morphant embryos (*n*=65); co-expressed lineage tracers are heavily concentrated in the ectopic structures (Fig. 5E).

Finally, in an attempt to demonstrate the specificity of the

Xema morpholinos, we engineered a 5′-Myc tagged Xema-fusion protein (Myc-Xema); this construct, which contains six tandem Myc epitopes upstream of the Xema coding sequence, was designed to bypass morpholino-mediated translational repression, which is most effective at or near the translational start site (Summerton, 1999). We find that co-injection of *Myc-Xema* RNA inhibits Xema knockdown-mediated mesendoderm formation, and rescues expression of *epidermal keratin* (Fig.
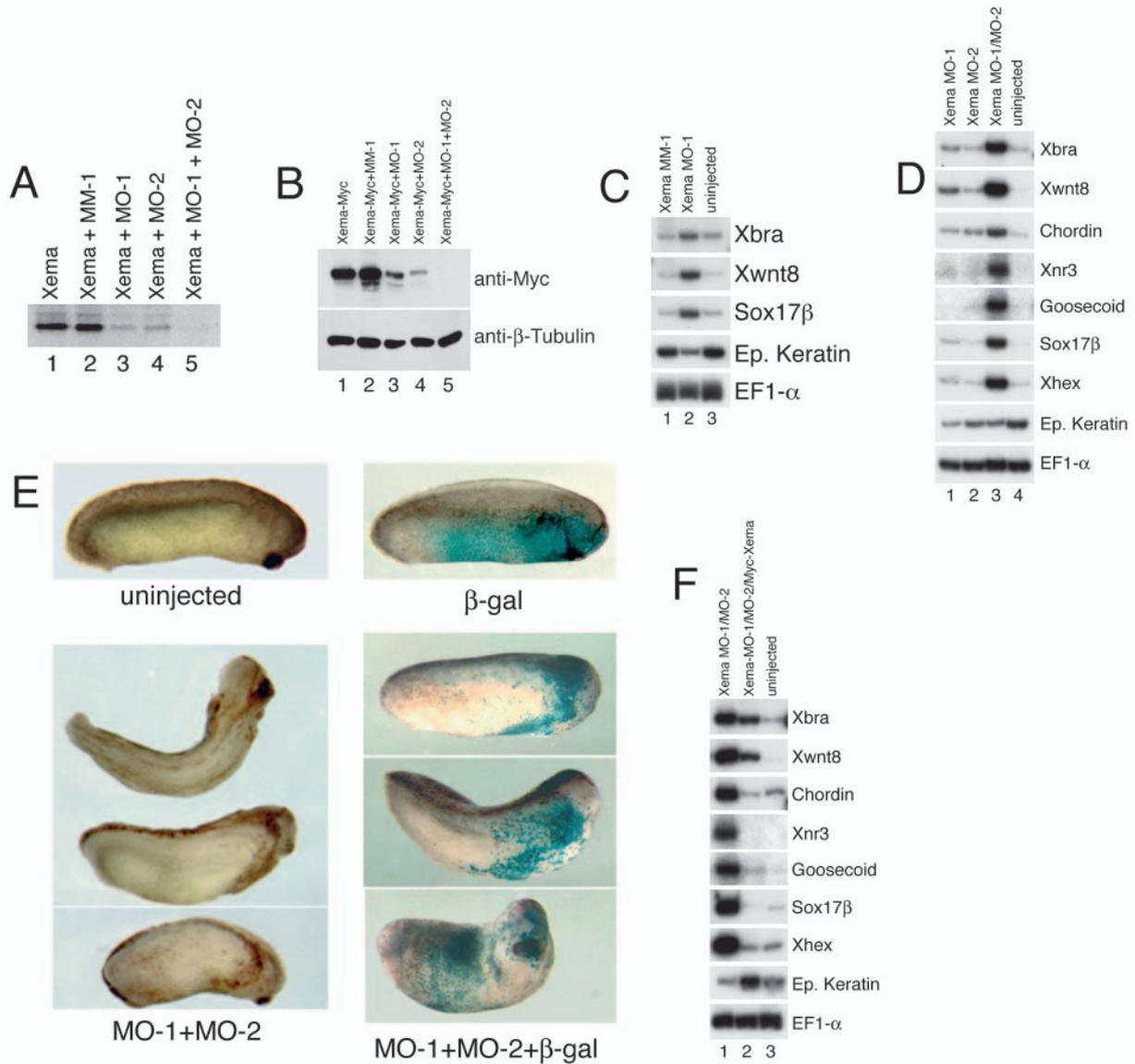

Fig. 5. Knockdown of Xema protein induces mesoderm formation. (A) Xema morpholinos block the translation of *Xema* RNA in vitro. Morpholino oligonucleotides (500 ng each) and *Xema* RNA (1 ng) were simultaneously added to a rabbit reticulocyte lysate mix (Promega), in the presence of [^{35}S]methionine. (B) The effect of Xema morpholinos on exogenous Xema protein levels. Western blot analysis of whole-cell lysates extracted from embryos injected with 1 ng *Xema-Myc* RNA; 10 ng MO-1, 10 ng MM-1, and/or 20 ng MO-2 were injected, as listed. (C) Mesoderm and endoderm are induced by injection of Xema MO-1 (10 ng), but not by Xema MM-1 (10 ng). RT-PCR analysis of animal cap explants from embryos injected with Xema morpholinos in the animal pole at early cleavage stages, dissected at blastula stages and harvested during midgastrula stages. (D) Xema MO-1 (10 ng) and MO-2 (20 ng) potently synergize to induce mesendoderm. (E) Co-expression of Xema MO-1 and MO-2 disrupts normal development. (Left column) Lower panels show embryos co-injected with 5 ng of MO-1 and 10 ng of MO-2 in the animal pole of early cleavage stage embryos. The top embryo is uninjected. Second embryo from top is a dorsal view; all other views are lateral; anterior is to right. Note anterior pigmented ectopic lateral structures. (Right column) Lower panels show embryos injected with 5 ng of MO-1, 10 ng MO-2 and 166 pg of *lacZ* RNA in the animal pole of early cleavage embryos. The top embryo is injected with 166 pg of *lacZ* RNA only. Note the presence of β-galactosidase staining (blue), primarily in the ectopic structures. All views are lateral; anterior is to right. (F) Rescue of Xema morpholino-mediated mesendoderm formation by injection of 1 ng *Myc-Xema* RNA; 10 ng MO-1 and 20 ng MO-2 were injected, as listed.

5F). Taken together, these data demonstrate that inhibition of Xema function stimulates mesendoderm formation in the animal pole, and strongly suggest that Xema is required to prevent inappropriate mesendodermal differentiation in the cells of the presumptive ectoderm.

Interactions between Xema and pathways regulating mesendoderm development

Inhibition of the signaling cascades triggered by either Fgf or the Activin/Nodal/Veg1 branch of the Tgfβ superfamily blocks the development of mesoderm in vivo (Amaya et al., 1991; Hemmati-Brivanlou and Melton, 1992). As Xema appears to play a role in preventing ectopic mesoderm formation in the ectoderm, it was of interest to determine the epistatic relationships between Xema and these signaling pathways. As described above, we found that Xema overexpression inhibits mesoderm induction by either Activin or Fgf (Fig. 3E,F); these results suggest that Xema may function at a convergence point downstream of the Tgfβ and Fgf pathways, or in an obligatory parallel pathway. We next addressed whether the activity of either pathway was required for mesoderm induction following a block to Xema function. Inhibition of Tgfβ signaling by expression of a truncated activin receptor (TAR) (Hemmati-Brivanlou and Melton, 1992), shown previously to act as a dominant-inhibitory reagent, does not potently inhibit mesoderm (*Xbra*, *Xwnt8*) induction by EnR-Xema (Fig. 6A, compare lanes 5 and 6), or mesoderm (*Xbra*, *Xwnt8*), dorsal endomesoderm (*chordin*), or endoderm (*Sox17β*) induction by Xema MO-1/MO-2 co-expression (Fig. 6B, compare lanes 1 and 2). We note that TAR expression did inhibit *Xbra* and, to a lesser extent, *Xwnt8* induction following Xema knockdown, in three out of 12 independent trials (data not shown); *chordin* and *Sox17β* expression were never inhibited by TAR in these assays. Inhibition of Fgf signaling by a truncated Fgf receptor (XFD) (Amaya et al., 1991), however, strongly inhibits *Xbra* and *Xwnt8* induction by both EnR-Xema (Fig. 6A; compare lanes 5 and 7) and by Xema MO-1/MO-2 co-expression (Fig. 6B; compare lanes 3 and 4). Fgf pathway inhibition, like Tgfβ pathway inhibition, does not affect the expression of dorsal endomesodermal (*chordin*) and endodermal (*Sox17β*) marker genes (Fig. 6B; compare lanes 3 and 4). These data suggest that Xema is not involved solely in the production of a secreted Tgfβ antagonist; furthermore, they suggest that Fgf signaling contributes to the capacity for mesodermal but not endomesodermal or endodermal development, normally suppressed by Xema, in the animal pole.

Discussion

In this study, we describe the characterization of Xema, a novel member of the Foxi-subclass of winged-helix transcription factors that is essential for the development of ectodermal fate. Xema expression is restricted to cells of the presumptive ectoderm in the *Xenopus* gastrula embryo; we demonstrate that Xema misexpression is sufficient to inhibit mesendoderm formation in a variety of assays, and that Xema appears to function as a transactivator in this regard. Crucially, we find that Xema activity is necessary for the suppression of ectopic mesendoderm in the animal pole. Our data are thus consistent with a model in which transcriptional activation by Xema either directly or indirectly stimulates an inhibitor of
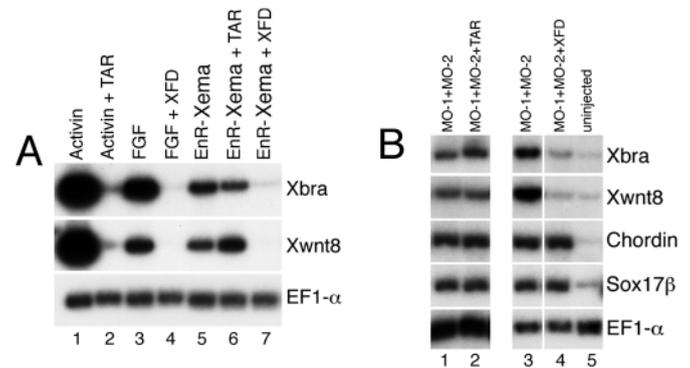

Fig. 6. Fgf signaling is required for mesoderm induction by either EnR-Xema or Xema morpholinos. (A) EnR-Xema activity is inhibited by co-expression of a truncated Fgf receptor (XFD), but is not inhibited by expression of a truncated activin receptor (TAR). RT-PCR analysis of animal cap explants dissected at blastula stages and harvested during midgastrula stages. *EnR-Xema* RNA (1 ng), alone or with either 4 ng XFD or 4 ng TAR, was injected at early cleavage stages, as indicated. Activin (0.5 ng/ml) or Fgf (10 ng/ml) was added to stage 9 animal caps, as indicated. (B) XFD inhibits Xema morpholino-mediated *Xbra* and *Xwnt8* expression. TAR did not inhibit *Xbra* or *Xwnt8* expression in nine out of 12 independent trials. Neither TAR nor XFD inhibit dorsal endomesodermal (*chordin*) or endodermal (*Sox17β*) marker induction by Xema morpholino injection; 4 ng TAR, 4 ng XFD, 10 ng MO-1 and 20 ng MO-2 were injected at early cleavage stages, as indicated.

mesendodermal fate in the presumptive ectoderm. This work, along with previous studies implicating a role for Hnf3β in mesoderm suppression in the frog and mouse (Perea-Gomez et al., 1999; Suri et al., 2004), suggests that Fox protein-mediated inhibition of ectopic germ layer development plays a critical role in establishing the early vertebrate body plan.

Our studies of Xema morphants have revealed an unexpected capacity for mesendodermal development in the cells of the presumptive ectoderm. We were surprised to find that morpholino-mediated Xema knockdown stimulates the expression of a wider range of mesendodermal marker genes than does injection of *EnR-Xema* RNA, particularly because the repressor construct might be expected to also block redundant activation by other Fox proteins on Xema-responsive genes in the animal pole. However, because EnR-Xema retains all native Xema sequence, including any putative Foxi transactivation domains, EnR-Xema probably functions as only a modest repressor of Xema target gene expression.

The source and nature of the 'mesendodermalizing' signal or signals suppressed by Xema are not known. One possibility is that decreased expression of Xema targets in the ectoderm leads to increased sensitivity of these cells to inducing signals from the vegetal pole. In *Xenopus*, the primary mesendoderm-inducing signal secreted by the vegetal pole is thought to be a Tgfβ ligand of the Nodal/Activin class, the expression of which is initiated at the start of zygotic transcription by the action of the transcription factor VegT (Whitman, 2001). Our data suggest that extracellular Tgfβ signaling is not the primary activity suppressed by Xema, although the occasional, albeit modest, effects seen following Tgfβ inhibition suggest some role for 'canonical' signals in the mesendodermalizing activity

unveiled by Xema knockdown. We also cannot exclude the possibility that the Xema morpholinos promote mesendodermal development indirectly, by affecting cell movements to 'reroute' marginal zone and vegetal pole cells to the animal pole.

Alternatively, or additionally, Xema may function to suppress a mesendodermalizing activity native to the animal pole. A role for such a signal is suggested by the demonstration that Fgf signaling is required for Xema knockdown-mediated ventral mesoderm development (Fig. 6), as it has been demonstrated that MAP kinase activity is present in the early gastrula ectoderm (LaBonne and Whitman, 1997); Xema may function in part to blunt the mesoderm-inducing potential of this activity. We note also that our studies with the truncated Activin receptor do not rule out the potential contribution of Tgfβ pathway stimulation to the mesendodermalizing signal downstream of the receptor and thus autonomous to the animal pole. Finally, it will be important to examine the relationship between Xema and the recently described 'rogue cells', found throughout the early gastrula embryo, that express one or more location-inappropriate molecular markers (Wardle and Smith, 2004). A subset of these cells is found in the animal pole at early gastrula stages and express mesodermal and/or endodermal markers, but they are largely undetectable by late gastrula stages (Wardle and Smith, 2004); Xema may thus be involved in the conversion and/or elimination of these cells during gastrulation.

Although our data suggest that transactivation of as yet uncharacterized Xema target genes leads to the suppression of ectopic mesendoderm, we do not yet have a clear sense of how Xema targets function to suppress inappropriate cell fate, nor, in a related issue, do we have a firm understanding of the eventual fate of the misexpressing cells. Our data suggest that, at least in the context of the dorsal marginal zone, Xema expression can promote the conversion of mesendoderm into ventral ectoderm; this effect is not seen in the ventral marginal zone or in Activin-treated animal caps. However, these assays all involve ectopic Xema and may not correlate precisely with endogenous Xema function in the presumptive ectoderm. Alternatively, Xema may induce or facilitate the elimination of ectopic mesendoderm during gastrulation. We have not observed any increase in TUNEL staining in embryos injected with *Xema* RNA, however, suggesting that mesendodermal suppression by Xema is not mediated via apoptosis (data not shown).

What relationships, if any, can be drawn between Xema and other proteins involved in the suppression of mesendodermal fate? The secreted Nodal antagonists, described earlier, are likely to play only a limited role in Xema-mediated germ layer inhibition. Sprouty proteins are Fgf antagonists involved in the regulation of a number of developmental processes (Kim and Bar-Sagi, 2004). *Xenopus* Sprouty2 antagonizes Fgf-mediated convergent extension movements, but appears to act independently of the Ras/MAPK branch of the cascade, required for mesoderm formation (Nutt et al., 2001). The C-Src Kinase (Csk) functions as an inhibitor of the Src family of non-receptor tyrosine kinases (Brown and Cooper, 1996); these latter proteins are involved in Fgf-mediated mesoderm induction in *Xenopus* (Weinstein et al., 1998). Strikingly, expression of a dominant-inhibitory form of *Xenopus* Csk (Xcsk) in the animal pole synergizes with sub-inducing doses of the Src kinase Laloo to form ventrolateral mesoderm and

secondary tails, suggesting that Xcsk is involved in suppressing mesoderm formation in the presumptive ectoderm (Song et al., 2001). Xcsk is widely expressed during early development (Song et al., 2001); thus, any regulation of Xcsk by Xema would probably be mediated post-transcriptionally. As Xema appears to function as a transcriptional activator, however, Xcsk is unlikely to be a direct Xema target. *Xenopus* Sip1 (Xsip1), is a direct repressor of *Xbra*, and thus represents a potential target and partial mediator of Xema (Lerchner et al., 2000; Papin et al., 2002). *Xsip1* transcripts are detected in the dorsal ectoderm of early gastrulae, suggesting that, at least at this stage, Xsip1 expression could be mediated by Xema (Eisaki et al., 2000; van Grunsven et al., 2000), either directly or through stimulation of the recently identified Churchill (Sheng et al., 2003). However, neither Xsip1 nor Churchill expression are stimulated by ectopic *Xema* RNA in the gastrula stage ectoderm, suggesting that mesoderm suppression by Xema is independent of Xsip1 (data not shown). Regardless, it is clear that Xema does not function solely as an Fgf pathway antagonist. While a role for Xema in the antagonism of intracellular Tgfβ signaling remains to be explored, a detailed appreciation of the mechanisms underlying germ layer suppression by Xema and other Fox proteins is likely to require the identification and characterization of direct transcriptional targets.

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