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XMAN1, an inner nuclear membrane protein, antagonizes BMP signaling by interacting with Smad1 in *Xenopus* embryos

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SUMMARY

A family of inner nuclear membrane proteins is implicated in gene regulation by interacting with chromatin, nuclear lamina and intranuclear proteins; however, the physiological functions of these proteins are largely unknown. Using a *Xenopus* expression screening approach with an anterior neuroectoderm cDNA library, we have identified an inner nuclear membrane protein, XMAN1, as a novel neuralizing factor that is encoded by the *Xenopus* ortholog of human *MAN1*. *XMAN1* mRNA is expressed maternally, and appears to be restricted to the entire ectoderm at the early gastrula stage, then to the anterior neuroectoderm at the neurula stage. XMAN1 induces anterior neural markers without mesoderm induction in ectodermal explants, and a partial secondary axis when expressed ventrally by dorsalizing the ventral mesoderm. Importantly, XMAN1 antagonizes bone morphogenetic protein (BMP) signaling downstream of its receptor Alk3, as judged by animal cap assays, in which XMAN1 blocks expression of downstream targets of BMP signaling (*Xhox3* and *Msx1*), and by luciferase reporter assays, in which XMAN1 suppresses BMP-dependent activation of the *Xvent2* promoter. Deletion mutant analyses reveal that the neuralizing and BMP-antagonizing activities of XMAN1 reside in the C-terminal region, and that the C-terminal region binds to Smad1, Smad5 and Smad8, which are intracellular mediators of the BMP pathway. Interference with endogenous XMAN1 functions with antisense morpholino oligos leads to the reduction of anterior neuroectoderm. These results provide the first evidence that the nuclear envelope protein XMAN1 acts as a Smad-interacting protein to antagonize BMP signaling during *Xenopus* embryogenesis.

Key words: *Xenopus laevis*, Neuralization, BMP signaling, Smad, Inner nuclear membrane protein, MAN1

INTRODUCTION

During early *Xenopus* embryogenesis, the neuroectoderm is induced from the ectoderm on the dorsal side of the gastrula embryo by signals from the underlying dorsal mesendoderm, a region known as the Spemann organizer. According to the current model (Harland and Gerhart, 1997), signals from the organizer uncover the default developmental condition of neural tissue with an anterior character by antagonizing bone morphogenetic proteins (BMPs), which function as epidermalizing factors. Concomitantly, the naive neuroectoderm is believed to be posteriorized and patterned along the anteroposterior axis by the trunk organizer. However, the molecular mechanisms of neuralization and anteroposterior patterning of the ectoderm have not yet been fully clarified.

It is widely accepted that inhibition of BMP signaling triggers neural induction in *Xenopus* embryogenesis. In recent years, a basic model for the signal transduction pathway from BMP receptors to the nucleus has been established (Heldin et al., 1997; Whitman, 1998; Massague and Chen, 2000; Wrana, 2000). BMP type II receptors form hetero-oligomeric complexes with type I receptors upon binding to BMPs. Subsequently, type II receptors phosphorylate and activate type I receptors, which, in turn, phosphorylate Smad1, Smad5 or Smad8. Phosphorylated Smads associate with Smad4 and translocate to the nucleus, where the complex binds to target genes together with an appropriate transcription factor(s). *Xvent1*, *Xvent2*, *Msx1* and *Xhox3* have been identified as BMP target genes in early *Xenopus* embryogenesis. In the case of *Xvent2*, OAZ has been identified as a Smad1-interacting transcription factor mediating the upregulation of *Xvent2* by BMP in the ventral region of *Xenopus* gastrulae (Hata et al., 2000).

Finding the molecules that modulate this signaling has helped our understanding of the mechanisms of neural induction by BMP antagonism (von Bubnoff and Cho, 2001). These molecules include extracellular antagonists (the Noggin, Chordin, Follistatin, and DAN family molecules), which bind BMPs to prevent activation of BMP receptors (Sasai et al., 1995; Zimmerman et al., 1996; Fainsod et al., 1997; Hsu et al., 1998; Iemura et al., 1998; Piccolo et al., 1999), and a pseudoreceptor (BAMBI), which acts as a dominant-negative BMP receptor (Onichtchouk et al., 1999). Molecules that interfere with Smad functions are also involved in BMP

antagonism. Smad6 blocks the BMP pathway by competing with Smad4 for binding to activated Smads (Hata et al., 1998), and Smad7 inhibits phosphorylation of Smad1 by interacting with activated BMP type I receptors (Souchelnytskyi et al., 1998). The Ski oncoprotein and Smad-interacting protein (SIP) bind to Smad1 (Verschueren et al., 1999; Wang et al., 2000) and suppress the expression of BMP target genes (Wang et al., 2000). Smurf1 and Smurf2, which are ubiquitin E3 ligases, mediate ubiquitination and degradation of Smad1 (Zhu et al., 1999; Zhang et al., 2001). Inhibition of the BMP pathway achieved by these molecules leads to neuralization of animal explants in *Xenopus* (Casellas and Brivanlou, 1998; Nakayama et al., 1998; Eisaki et al., 2000; Harland, 2000), and is also thought to be crucial for controlling the expression levels of target genes for BMP signaling.

A *Xenopus* expression cloning system has been one of the most powerful methods used to identify molecules that are involved in a variety of developmental processes (Grammer et al., 2000). Although many novel genes have been successfully identified with this system, most of this work has been carried out using cDNA libraries prepared from whole embryos containing different cell populations, which results in the dilution of gene contents of interest and hence increases in the number of genes needed to be screened. We therefore made an anterior neuroectoderm (ANE) cDNA library from *Xenopus* late gastrulae to increase cloning efficiency, and screened it for genes that are involved in neuralization and neural patterning. We focused on the ANE to clarify the following features of ANE: (1) it might contain a source of secreted factors responsible for 'homeogenetic induction', neural induction by the neural plate (Mangold and Spemann, 1927; Servetnick and Grainger, 1991); (2) the ANE should have its own neuralizing activity as a default state after suppressing BMP signaling; and (3) the ANE may have self-regionalizing activity to give rise to brain structures from the forebrain to midbrain, and thus generate a wide variety of cell populations during neurulation.

To date, several inner nuclear membrane proteins are known, including lamin B receptor (LBR), lamina-associated polypeptide 1 (LAP1), LAP2, emerin, MAN1 and nurim (Worman and Courvalin, 2000). Most of these have been shown to interact with the nuclear lamina and chromatin. In addition, LAP2, emerin and MAN1 share a conserved domain, called the LEM domain, near their N termini (Lin et al., 2000). The LEM domains of LAP2 and emerin have been shown to bind barrier-to-autointegration factors (BAFs) (Furukawa, 1999; Lee et al., 2001; Shumaker et al., 2001), which bind to DNA to bridge between the LEM domain-containing protein and DNA, and are thought to be involved in chromatin decondensation and nuclear assembly (Segura-Totten et al., 2002). However, the physiological functions of inner nuclear membrane proteins have not been fully elucidated, especially in terms of signal transduction pathways.

We report here a novel neuralizing factor, XMAN1, the ortholog of human MAN1, identified by an expression screening with the ANE cDNA library. We also present evidence that XMAN1 acts as a Smad-interacting protein and could be involved in neural development by antagonizing BMP signaling. This provides new insights into the molecular mechanisms of neural induction and the regulation of BMP signaling, as well as elucidating the role of an inner nuclear protein in gene regulation.

MATERIALS AND METHODS

Xenopus embryo manipulation

Artificial fertilization and culture of embryonic tissues were performed as described previously (Kay and Peng, 1991). Embryos were staged according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1967). Animal cap and ventral marginal zone assays were performed as previously described (Taira et al., 1994; Osada and Wright, 1999).

Construction and functional screening of an ANE library

About 500 pieces of anterior neural plates were dissected from stage 12-12.5 embryos. The neuroectoderm layer was separated from the underlying mesendoderm layer in 1× modified Barth's solution containing 1-2 mg/ml collagenase (Shibata et al., 2001). After poly $(A)^+$ RNA selection by an oligo(dT) cellulose column, a cDNA library was made with the SuperScript plasmid system for cDNA synthesis and plasmid cloning (Invitrogen) and the pCS105 vector. Two hundred pools, each containing about 200 independent cDNA clones, were prepared, and capped RNA was synthesized from each pool as previously described (Taira et al., 1994). Capped RNA (10 ng) was injected into the animal pole region of one-cell stage embryos. Animal caps were dissected at stages 8-9 and cultured until siblings reached stage 25.

cDNA cloning and plasmid constructs

Screening of a stage 30 head cDNA library for a full-length clone of *XMAN1* was carried out using a standard method. 5′-RACE was performed using the 5′-full RACE core set (Takara) and poly$(A)^+$RNA from stage 12.5 embryos. Constructs for Myc-tagged full-length (amino acids 1-781, MT-XMAN1), ΔLEM (amino acids 44-781, MT-ΔLEM), ΔNT (amino acids 450-781, MT-ΔNT), ΔCT (amino acids 1-581, MT-ΔCT) and CT (amino acids 582-781, MT-CT) of XMAN1 were made using PCR and the pCS2+MT vector. MT-ΔRNP1, MT-ΔRNP2 and MT-ΔRNP1+2 constructs were made with the GeneEditor in vitro site-directed mutagenesis system (Promega) using MT-XMAN1 as a template. HA-tagged mouse Smad3, Smad5, Smad6, Smad7 and Smad8 were made by transferring the inserts from pcDEF3 constructs to the pCS2+HA vector.

Whole-mount in situ hybridization and histological studies

Whole-mount in situ hybridization was performed according to Harland (Harland, 1991) using an automated system (AIH-101, Aloka). An antisense *XMAN1* RNA probe was synthesized by transcribing a *Not*I-linearized clone pXMAN1-11 (the longest clone found after library screening) with T7 RNA polymerase. Other RNA probes were synthesized according to plasmid providers. Some stained embryos were embedded in paraffin wax and sectioned at 10-15 μm.

RT-PCR

Animal caps and marginal explants were dissected when siblings reached the stages indicated, and RT-PCR was carried out as previously described (Osada and Wright, 1999), except that DNA amplification was achieved non-radioactively. PCR products were analyzed on 2% agarose gels in the presence of ethidium bromide; images of fluorescent DNA fragments were digitally recorded by GelPrint 2000i (Genomic Solutions), and black/white inversion was performed using PhotoShop software (version 5.5, Adobe Systems). Primers for XMAN1 were: forward, 5′-CAAATTTGCAGTCATGC-TCT-3′; reverse, 5′-AAAATAAGTGGGGCCCTATG-3′. In several experiments, real-time RT-PCR was carried out using ABI PRISM 7000 (Applied Biosystems) with Taq-Man probes (Table 1) that allowed the detection of only primer-specific PCR products. Each real-time PCR was performed in triplicate. EF-1α was used as an internal control and each bar was normalized to the level of EF-1α expression.

Luciferase assays

An Xvent2 promoter luciferase (Xvent2-Luc) or a control pGL3-Luc construct (100 pg/embryo) was injected together with a *Renilla* luciferase plasmid (pRL-TK; 2 pg/embryo) and RNA mixtures into the animal regions of one-cell stage embryos. Animal caps were dissected at stages 8-9, and pools of three explants were collected in triplicate for each injection mixture at stage 10.5. Luciferase assays were done using the double luciferase reporter assay system (Promega). Each pool was homogenized in 50 μl of 1× lysis buffer (kit reagent) by vortexing and cleared by microcentrifugation. The supernatant (10 μl) was assayed in 50 μl of assay mixture, and luciferase activity was measured for 10 seconds with a luminometer (Berthold).

Immunofluorescence microscopy

COS-7 cells were grown in Dulbecco's modified Eagle's (DME) medium containing 10% fetal bovine serum. For transfection, cells were grown to almost 80% confluence and transfected using FuGENE 6 Transfection Reagent (Roche). After 24-hour incubation, transfected cells were washed twice with phosphate-buffered saline (PBS) and fixed with 2% paraformaldehyde in PBS for 15 minutes at room temperature. Fixed cells were then permeabilized with 0.5% Triton-X 100 in PBS for 10 minutes, blocked in PBS containing 10% lamb serum for 1 hour at room temperature, and incubated with primary antibody in Tris-buffered saline (10 mM Tris-HCl, pH 7.5, 150 mM NaCl) containing 0.01% Tween 20 (TBS-T) for 1 hour at 37°C. Anti-FLAG M2 monoclonal antibody (Sigma) and anti-Myc monoclonal antibody 9E10 (Biomol) were used as primary antibodies at a dilution of 1:1000. After washing three times with TBS-T, the cells were incubated with secondary antibody Alexa 488-conjugated goat anti-mouse IgG (Molecular Probes) at a concentration of 5 μg/ml. Nuclei were visualized by co-transfecting the pDsRed2-Nuc vector (Clontech). Protein localization was examined by laser-scanning confocal microscopy using LSM5 Pascal (Zeiss).

GST pull-down assays

The C-terminal region of XMAN1 was subcloned into the pGEX-6P-1 vector to produce the GST-XMAN1-CT fusion protein. [^{35}S]methionine-labeled proteins were synthesized with the TNT SP6-coupled reticulocyte lysate system (Promega). Purification of GST and GST-XMAN1-CT proteins, and GST pull-down assays were as previously described (Hiratani et al., 2001).

Western blotting and immunoprecipitation experiments

Capped RNAs for Myc-tagged XMAN1 constructs were co-injected with or without that for HA-tagged XSmad1 into the animal regions of two-cell stage embryos. Injected embryos were cultured until the mid gastrula stage 11, homogenized in 1× lysis buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 5 mM EDTA, 0.5% Nonidet P-40, 1 mM PMSF, 1 mg/ml aprotinin; 100 μl per embryo), and microcentrifuged. The lysate (250 μg protein) was incubated with 1 μg of anti-HA polyclonal antibodies (Santa Cruz) for 1 hour at 4°C, then incubated with protein G-agarose (Roche) for another hour. Immunoprecipitates were washed five times in 1× lysis buffer, boiled in SDS sample buffer, and analyzed by western blotting with anti-Myc antibody 9E10 (1:2000, BIOMOL) and horseradish peroxidase-conjugated anti-mouse IgG antibody (1:2000, Amersham Pharmacia). Probing with a monoclonal anti-β-tubulin antibody (1:2500, Sigma) was for loading control. Detection was carried out using the ECL+Plus kit (Amersham Pharmacia).

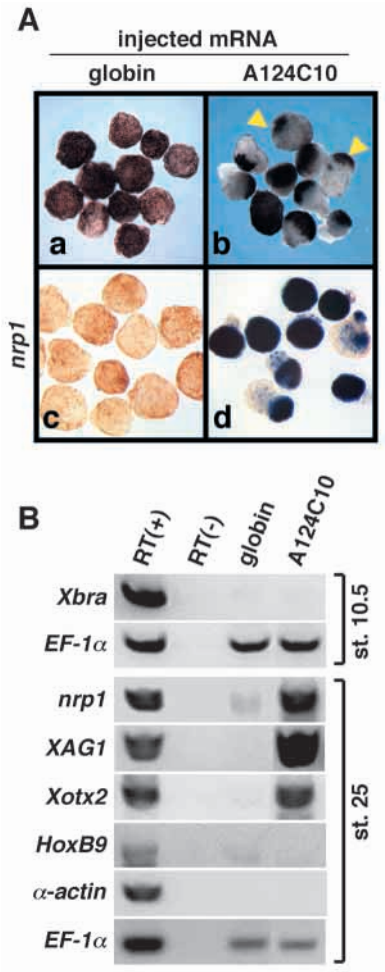

Fig. 1. Isolation of a novel neuralizing factor. (A) Animal caps expressing β-globin (a,c) and A124C10 (b,d) at the equivalent of stage 25. Whole-mount in situ analysis for *nrp1* expression was carried out in c,d. Arrowheads, cement glands. (B) RT-PCR analysis for neural marker expression in β-globin or A124C10 expressing animal caps at early gastrula (stage 10.5) and tailbud (stage 25) stages. RT(+) and RT(–) indicate whole-embryo RNA transcribed with or without reverse transcriptase, respectively. EF-1α was used as a loading control. Doses of injected mRNA (pg/embryo): *β-globin*, 500; A124C10, 500.

Table 1. PCR primer pairs and TaqMan probes used for real-time PCR with PRISM 7000

Gene	PCR primers	TaqMan probes
nrp1	F: 5′-TGTCACCAACAGGATCTGTGAGA-3′ R: 5′-CCTATTCCCAGCATGAAAGCAT-3′	5′-CGATCTCGGGTCATGCCCTATGGAA-3′
Xotx2	F: 5′-ACGCAGGCATCAGGGTACA-3′ R: 5′-CCACAGTCCATACCCCCAAA-3′	5′-CCAAGGATATGCAGGCTCAACATCC-3′
EF-1α	F: 5′-CCCTGCTGGAAGCTCTTGAC-3′ R: 5′-GAGGCAGACGGAGAGGCTTAT-3′	5′-TTTGCCACCATCTCGCCCAACC-3′

F, forward; R, reverse

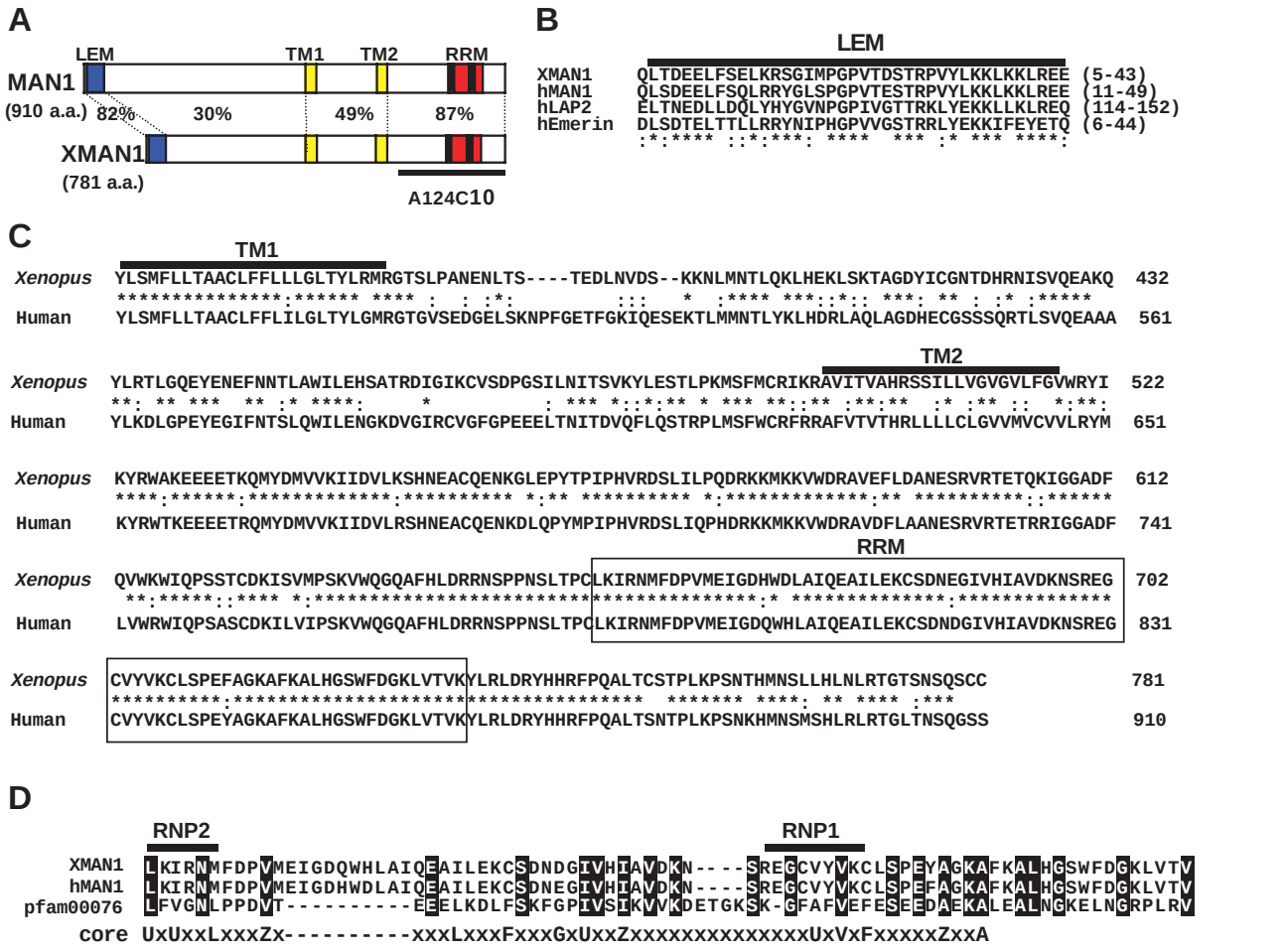

Fig. 2. Structural features of XMAN1. (A) Schematic structural comparison of human (Accession Number, AF112299) and *Xenopus* MAN1 (Accession Number, AB100267). LEM domains (blue), putative transmembrane domains (TM, yellow), and RNA recognition motif (RRM, red) are indicated. RNP2 and RNP1, conserved regions among RRMs, are shown as black boxes. A black bar represents the coding region of A124C10. The total numbers of amino acid residues of each protein and sequence identities of each domain between the two proteins are shown. (B) The sequence of the LEM domain of *Xenopus* MAN1 in comparison with those of human MAN1, LAP2 and emerin. Asterisks and colons indicate identical and homologous amino acid residues, respectively, conserved among at least three molecules. (C) Sequence comparison between human and *Xenopus* MAN1 from the first transmembrane domain. Asterisks and colons are indicated as above. RRM domains are boxed. (D) Alignment of putative RRMs of human and *Xenopus* MAN1. Pfam00076 represents an RRM in a database for multiple sequence alignments (Bateman et al., 2002). A core sequence for RRM is reported previously (Birney et al., 1993). U, uncharged; Z, uncharged, serine or threonine; x, any amino acid residues.

Antisense morpholino experiments

Antisense morpholino oligos complementary to *XMAN1* mRNA (XMAN1-MO, 5′-GCCGCCATTTTGACCACTCGGT-3′), its four-base-mismatched control oligos (XMAN1-4mmMO, 5′-GCgGCgA-TTTTGACCAgTCcGT-3′; where lower cases indicate mismatch mutations) and nonspecific control oligos were obtained from Gene Tools.

RESULTS

Functional screening for neuralizing factors

We screened 200 pools (approximately 40,000 independent clones) for activity to induce a pan-neural marker, *nrp1*, in animal caps by RT-PCR or whole-mount in situ hybridization. Twenty-one of the pools showed such neuralizing activity. These were further sib-selected, and the single clones that were responsible for neuralizing the ectoderm were isolated. We found that these clones included *BF2* (Mariani and Harland, 1998), *Xsox3* (Penzel et al., 1997), *Geminin* (Kroll et al., 1998), *SoxD* (Mizuseki et al., 1998b), *Xiro3* (Bellefroid et al., 1998) and *Zic3* (Nakata et al., 1997), which are all expressed in the anterior neuroectoderm and possess neuralizing activity, indicating that our strategy worked well. Among these, we focused on clone A124C10. Although uninjected animal caps developed into atypical epidermis (Fig. 1A, part a) without expression of *nrp1* (Fig. 1A, part c), A124C10 induced the development of cement glands (Fig. 1A, part b) and *nrp1* expression (Fig. 1A, part d). RT-PCR analysis with animal caps expressing A124C10 showed that it induces the anterior markers, *XAG1* (cement gland) and *Xotx2* (forebrain), but not the mesodermal markers, *Xbra* and *α-actin*, or posterior neural markers, *En2* (midbrain/hindbrain boundary), *Krox20* (hindbrain) or *HoxB9* (spinal cord) (Fig. 1B; data not shown).

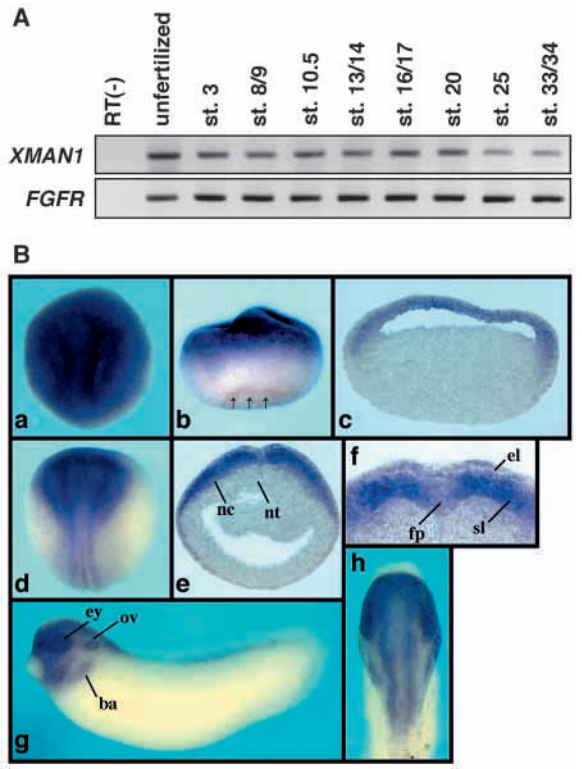

Fig. 3. Temporospatial expression of *XMAN1*. (A) Analysis of temporal expression pattern of *XMAN1* by RT-PCR with RNA from various developmental stages. FGFR, a loading control. (B) Whole-mount in situ analysis of *Xenopus* embryos at early gastrula (a-c), neurula (d-f) and tadpole (g,h) stages. c and e show cross-sections of b and d, respectively. f represents a close view of the dorsal region of e. h shows a dorsal view of the head region of g. Arrows, dorsal blastopore groove. nc, neural crest; nt, notochord; fp, floor plate; el, epithelial layer; sl, sensory layer; ey, eye; ov, otic vesicle; ba, branchial arches.

Whole-mount in situ analysis also showed the same results (not shown). Thus, A124C10 induces neural tissue with an anterior character without the concomitant induction of mesoderm in animal caps.

A124C10 encodes the C-terminal region of MAN1

Sequence analysis indicated that A124C10 shows the highest similarity to the C-terminal region of human MAN1. MAN1 was identified as one of three 'MAN antigens' localized exclusively to the nuclear membrane recognized by auto-antibodies from an individual suffering collagen vascular disease, and encodes for an inner nuclear membrane protein (Paulin-Levasseur et al., 1996; Lin et al., 2000). We isolated a full-length clone for the *Xenopus* ortholog of *MAN1* by screening a stage 30 head cDNA library using A124C10 as a probe and by 5′-RACE with poly $(A)^+$ RNA from stage 12.5 *Xenopus* embryos. A BLAST search with the sequence of the full-length clone again showed the highest similarity to human MAN1: 56% identity overall and 87% identity in the C-terminal region (Fig. 2A). Structurally, XMAN1 has an LEM domain in the N-terminal region (Fig. 2B) and two putative transmembrane domains, as does human MAN1 (Fig. 2C). The sequences of the first transmembrane domains are highly

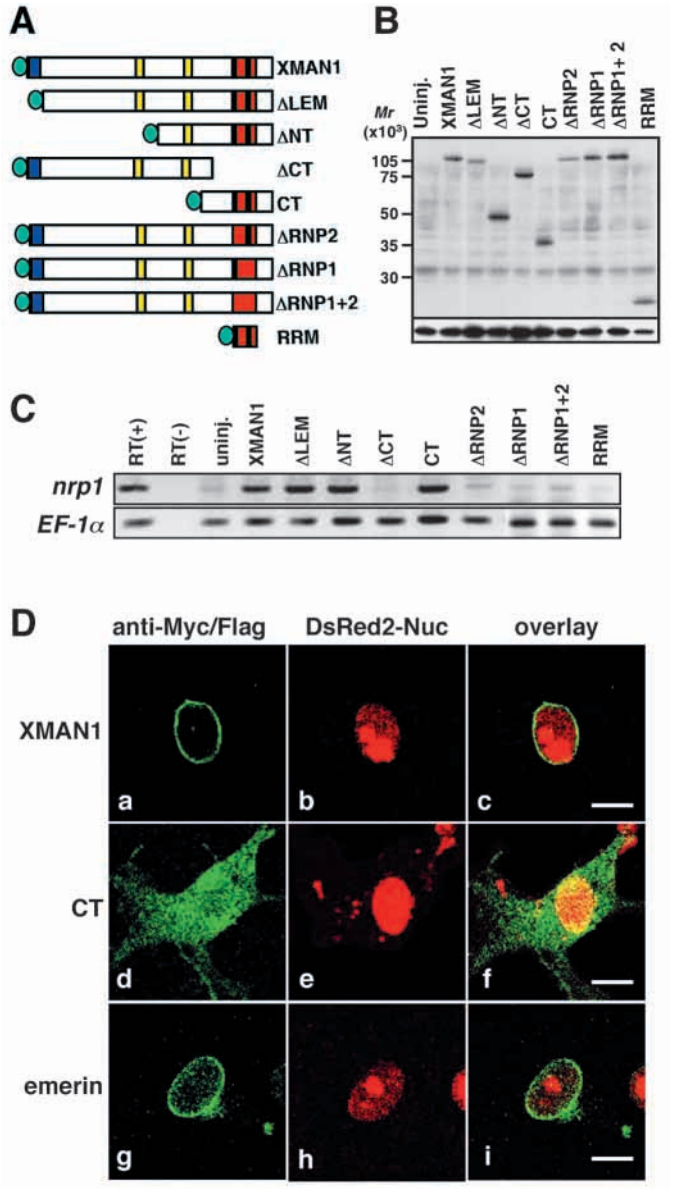

Fig. 4. Neuralizing activity of XMAN1 resides in the C-terminal region. (A) Schematic representation of XMAN1 deletion mutants. LEM domains, transmembrane domains and RRMs are depicted as in Fig. 2A. Circles indicate Myc tags. (B) Western blot for analyzing the expression level of the XMAN1 deletion mutants. Extracts prepared from stage 11 gastrula embryos expressing XMAN1 deletion mutants were immunoblotted with anti-Myc antibody (upper panel) or anti-β-tubulin antibody (lower panel; loading control). (C) Mapping of neuralizing activity of XMAN1. RT-PCR analysis for *nrp1* was performed with animal caps at the equivalent of the tailbud stage 25. Doses of injected mRNA: 500 pg per embryo. (D) Confocal microscopic analysis of the subcellular localization of Myc-tagged XMAN1 (a-c), Flag-tagged XMAN1-CT (d-f) and Myc-tagged human emerin (g-i) expressed in COS-7 cells (green). Nuclei (red) were visualized by co-transfecting with the DsRed2-Nuc vector. Scale bars: 10 μm.

conserved between human and *Xenopus* (92% identity), whereas those of the second domains are considerably more diverse (39% identity). Thus, we considered this clone to be

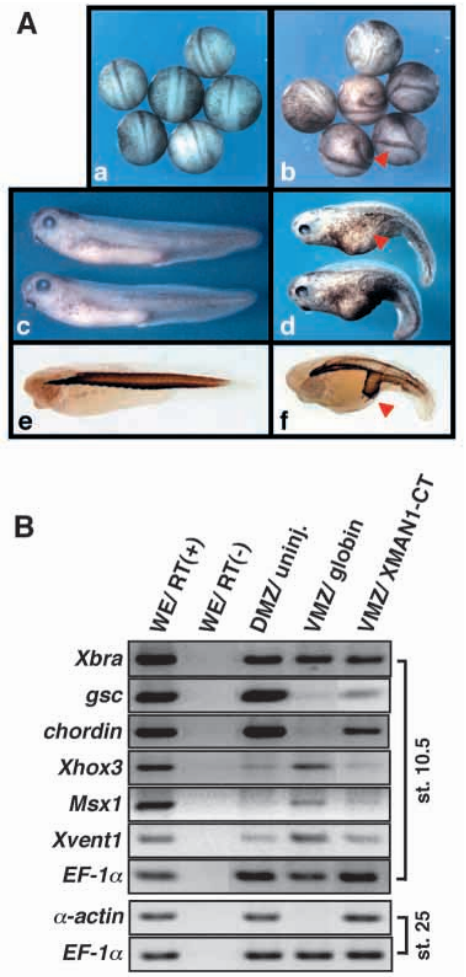

Fig. 5. XMAN1 dorsalizes the ventral mesoderm. (A) Embryos were injected with *β-globin* (a,c,e) or XMAN1-CT (b,d,f) mRNA into the ventral marginal zone at the four-cell stage and observed at stage 20 (a,b) or stage 35 (c-f). Somites were stained with 12/101 antibody (e,f). Arrowheads, secondary axes. (B) RT-PCR analysis of RNA from *β-globin* or XMAN1-CT mRNA (500 pg per embryo)-injected ventral marginal zones for expression of pan-mesodermal (*Xbra*), dorsal (*gsc*, *chordin* and α-*actin*) and ventral (*Xhox3, Msx1* and *Xvent1*) mesodermal markers.

the *Xenopus* ortholog of *MAN1* (*XMAN1*). A detailed sequence analysis led us to identify an RNA-recognition motif (RRM) in the C-terminal region (Fig. 2C,D). This contains two conserved regions, RNP2 and RNP1, and is believed to associate with RNA (Birney et al., 1993), although the RRM in a particular protein is also involved in protein/protein interactions (Dye and Patton, 2001).

Expression profile of *XMAN1*

Developmental expression patterns of *XMAN1* were analyzed by RT-PCR using RNA isolated from different stages of embryos and by whole-mount in situ hybridization. Fig. 3A shows that *XMAN1* transcripts are present as maternally expressed genes, and the expression levels are relatively constant throughout early embryogenesis. At the early gastrula stage, *XMAN1* is mainly expressed in the entire animal hemisphere (Fig. 3B, parts a-c). During neurulation, the expression of *XMAN1* becomes restricted to the ANE (Fig. 3B, part d), from where clone A124C10 was derived. Examination of a section of a stage 16 embryo revealed that *XMAN1* is expressed in the sensorial layer of the neuroectoderm and the neural crest, weakly in the epithelial layer and presumptive floor plate, but not in the notochord (Fig. 3B, parts e,f). At the tailbud stage, *XMAN1* is expressed in various regions including the anterior central nervous system (CNS), eyes, otic vesicles and bronchial arches (Fig. 3B, parts g,h). A sense *XMAN1* probe gave no signals (data not shown). The expression of *XMAN1* in the ectoderm and neuroectoderm region is consistent with its neuralizing activity.

XMAN1 neuralizing activity resides in the C-terminal region

We made a series of deletion mutants of *XMAN1* to map the region that is necessary for its neuralizing activity (Fig. 4A). All Myc-tagged constructs were effectively translated when expressed in *Xenopus* embryos (Fig. 4B). Deletion constructs of the LEM domain (MT-ΔLEM) and the N-terminal region including the first putative transmembrane domain (MT-ΔNT) still have neuralizing activity comparable with that of wild type (Fig. 4C). However, a mutant that lacks the 200 C-terminal residues (MT-ΔCT) did not neuralize the ectoderm, indicating that XMAN1 neuralizing activity resides in the C-terminal region. This is consistent with our initial results showing that clone A124C10, an equivalent of the C-terminal region of XMAN1, also has neuralizing activity. The C-terminal region is highly conserved between human and *Xenopus* (87% identity; Fig. 2A,C), suggesting evolutionary conservation of this activity. Because the C-terminal region contains an RRM, we examined whether this is necessary for the activity. Injecting mutants that lack either RNP2 or RNP1 (MT-ΔRNP2 or MT-ΔRNP1, respectively), or both (MT-ΔRNP1+2) abolished neuralizing activity. The RRM alone (MT-RRM) failed to neuralize the ectoderm, suggesting that the RRM is necessary but insufficient for the neuralizing activity of XMAN1.

We then compared the subcellular localization of the wild-type XMAN1 and the C-terminal fragment of XMAN1 (CT). COS-7 cells were transfected with tagged XMAN1, CT or human emerin (an inner nuclear membrane protein used as a control), and their subcellular localizations were analyzed using a confocal microscope. As shown in Fig. 4D, XMAN1 was localized to the nuclear rim (parts a-c), as has been shown for human MAN1 (Lin et al., 2000). This localization pattern is very similar to that of human emerin (parts g-i). By contrast, CT, which lacks the putative transmembrane domains of XMAN1, was not localized to the nuclear envelope but was ubiquitously distributed within the cell (parts d-f). Myc-tagged full-length XMAN1 protein expressed in the animal cap cells was also localized to the nuclear membrane (not shown). The neuralizing factors identified so far have been categorized into several groups, including secreted, cytoplasmic, or transcription factors (Harland, 2000). Our data indicate that XMAN1 is the first neuralizing molecule localized to the nuclear envelope.

XMAN1 dorsalizes the ventral mesoderm

Several neuralizing factors can induce a secondary axis when expressed in the ventral marginal zone. Fig. 5A shows that this

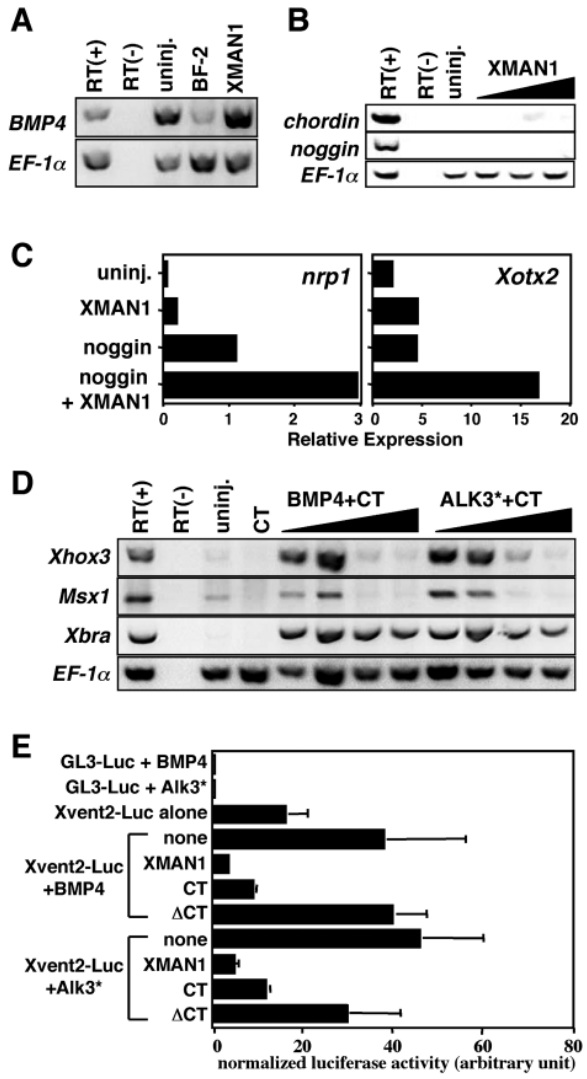

Fig. 6. XMAN1 antagonizes the BMP pathway. XMAN1 does not suppress the expression of *BMP4* (A) or induce BMP antagonists (B). RT-PCR analyses for *BMP4* (A), *noggin* and *chordin* (B) with animal caps at the equivalent of stage 10.5. (C) XMAN1 acts synergistically with Noggin in neural induction. Real-time RT-PCR analyses for *nrp1* and *Xotx2* with animal caps at the equivalent of stage 25. *EF-1*α was used as an internal control, and each bar was normalized to the level of *EF-1*α expression. Representative data from three independent experiments are shown. Doses of injected mRNA (pg/embryo): *XMAN1*, 200; *noggin*, 2. (D) XMAN1 blocks the BMP pathway. RT-PCR analysis for *Xhox3*, *Msx1* and *Xbra* with animal caps at the equivalent of stage 10.5. (E) Suppression of BMP-dependent Xvent2-Luc activation by XMAN1 constructs. Alk3*, activated form of type I BMP receptor; GL3-Luc, control luciferase construct.

is also the case for XMAN1. XMAN1-CT induced a secondary axis (parts b,d) that contained muscle, but not the notochord (part f and data not shown). We next examined dorsal marker gene expression by ventral marginal zone assays. In XMAN1-CT mRNA-injected marginal zones, all the dorsal markers (*gsc*, *chordin* and *α-actin*) were elevated, whereas the ventral markers (*Xhox3*, *Msx1* and *Xvent1*) were suppressed, and the level of a pan-mesodermal marker (*Xbra*) was unchanged (Fig. 5B). These results indicate that XMAN1-CT dorsalizes the ventral mesoderm without changing the fate of the mesoderm.

XMAN1 inhibits BMP signaling

The observation that XMAN1 neuralizes the ectoderm and dorsalizes the ventral mesoderm suggests that it represses BMP signaling. To test this possibility, we first examined whether XMAN1 suppresses *BMP4* expression. Fig. 6A shows that, although BF-2 suppresses *BMP4* expression in animal caps as reported previously (Mariani and Harland, 1998), XMAN1 did not affect *BMP4* expression. We next examined whether neuralizing activity of XMAN1 is a secondary effect of the induction of the BMP4 antagonists, Chordin or Noggin. As shown in Fig. 6B, XMAN1 did not induce the expression of *noggin* or *chordin* in animal caps, even at high doses, indicating that XMAN1 activity is not mediated by Chordin and Noggin.

Because maternal *XMAN1* mRNA is present before *noggin* and *chordin* expression starts, it is reasonable to speculate that XMAN1 sensitizes the ectoderm to respond to the BMP antagonists secreted from the organizer. Thus, we examined whether XMAN1 acts synergistically with Noggin in neuralizing animal caps using real-time PCR (Fig. 6C). When expressed alone, low amounts of *XMAN1* (200 pg) or *noggin* (2 pg) mRNA produced only weak expression of the neural markers, *nrp1* and *Xotx2*, in animal caps. When co-expressed, the expression of these markers was enhanced, suggesting that XMAN1 and Noggin act co-operatively in neural induction.

To investigate whether XMAN1 blocks the signal transduction of BMP, we co-injected mRNA for *XMAN1* with that for *BMP4* or an activated form of type I BMP4 receptor, Alk3*. BMP4 or Alk3* alone induced the downstream targets of BMP signaling, *Xhox3* and *Msx1* (Fig. 6D). The induction of these markers was inhibited by XMAN1-CT in a dose-dependent manner, whereas the induction of *Xbra* by BMP4 and Alk3* was less sensitive to the inhibitory effects of XMAN1-CT. XMAN1-CT at higher doses (more than 500 pg per embryo) and wild-type XMAN1 at moderate doses (100-250 pg per embryo) almost completely inhibited BMP-induced *Xbra* expression (not shown). These results indicate that XMAN1 antagonizes BMP4 signaling downstream from the receptor.

The above results were supported by a reporter assay using a BMP-responsive *Xvent2*-luc construct (Fig. 6E). Because BMP proteins are present in ectodermal explants (Hemmati-Brivanlou and Thomsen, 1995), Xvent2-luc is activated at a certain level without stimulation with BMP4 or Alk3*. Injection of *BMP4* or Alk3* mRNA enhanced this basal level by two- to threefold. By contrast, co-expression of the full-length or the C-terminal constructs of XMAN1 (MT-XMAN1 or MT-CT), but not a C-terminal truncated construct (MT-ΔCT), suppressed the reporter activity even below the basal level. Repeatedly, MT-XMAN1 appears more efficient in suppressing Xvnet2-Luc than MT-CT, suggesting that proper intracellular localization is necessary for the full activity of XMAN1 (see Fig. 4D).

XMAN1 binds to BMP-responsive Smads

Several Smad-interacting proteins have been shown to interfere with TGFβ signaling pathways (Heldin et al., 1997; Massague and Chen, 2000; Wrana, 2000). Thus, we next examined whether XMAN1 physically associates with BMP-responsive

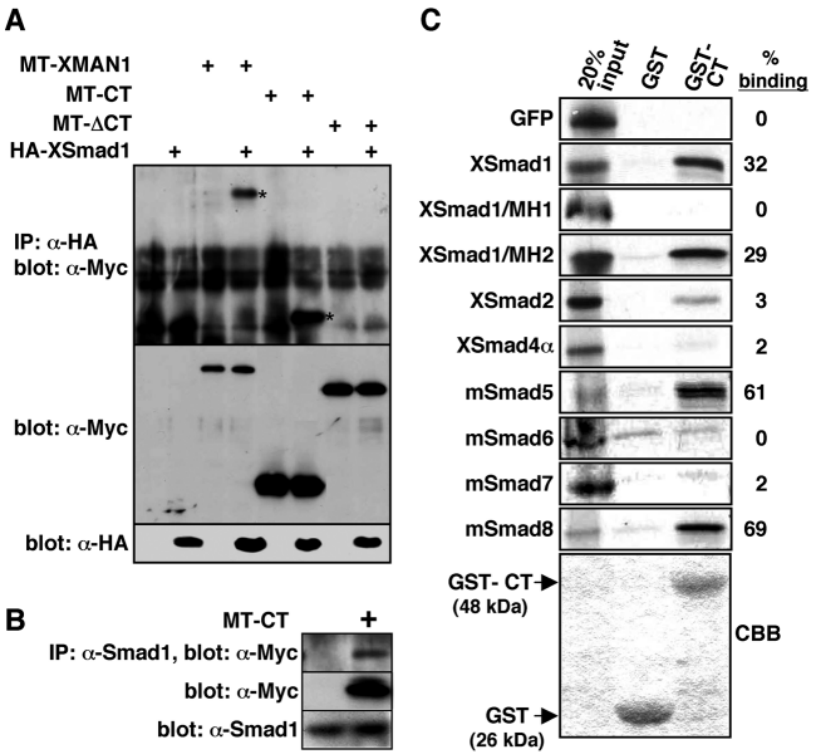

Fig. 7. XMAN1 preferentially associates with BMP-responsive Smads. (A) XMAN1 associates with XSmad1 in vivo. Myc-tagged wild-type (MT-XMAN1), C-terminal (MT-CT) and N-terminal (MT-ΔCT) constructs of XMAN1 were co-expressed with HA-tagged XSmad1 in *Xenopus* embryos as indicated. Extracts prepared from stage 10.5 embryos were immunoprecipitated with an anti-HA antibody. The precipitates were analyzed by western blotting with an anti-Myc antibody (upper panel). Middle and lower panels show the expression levels of XMAN1 and XSmad1 proteins, respectively. (B) XMAN1 associates with endogenous XSmad1. Extracts prepared from stage 10.5 embryos injected with or without MT-CT were immunoprecipitated with anti-Smad1 antibodies, and the precipitates were immunoblotted with anti-Myc antibody (upper panel). Middle and lower panels show the expression levels of MT-CT and endogenous XSmad1 proteins, respectively. (C) XMAN1 interacts preferentially with BMP-responsive Smads in vitro by GST pull-down assays. [^{35}S]Met-labeled in vitro translated Smad and GFP products were incubated with purified GST or GST-XMAN1-CT and subjected to SDS-PAGE. % binding, percentage of the intensity of each band in the lane of GST-CT against the corresponding total input.

Smads by co-immunoprecipitation and GST pull-down assays. MT-XMAN1, MT-ΔCT or MT-CT was co-expressed in embryos with HA-tagged *Xenopus* Smad1 (XSmad1), a transducer of BMP signaling. XSmad1 was then immunoprecipitated with an anti-HA antibody, and subsequently the precipitates were analyzed by western blotting with an anti-Myc antibody. As shown in Fig. 7A, MT-XMAN1 and MT-CT, but not MT-ΔCT were co-immunoprecipitated with XSmad1, indicating that XMAN1 interacts with XSmad1 through its C-terminal region in the embryo. MT-CT was also co-immunoprecipitated with endogenous XSmad1 (Fig. 7B).

To examine the interaction between XMAN1 and Smads more directly, and to address the specificity of association with Smads, we performed GST pull-down assays. Purified GST alone as negative control or a GST fusion protein with the C-terminal region of XMAN1 (GST-XMAN1-CT) was incubated with either one of the ^{35}S-labeled Smads, or with the MH1 or MH2 domain of XSmad1. As shown in Fig. 7C, GST-XMAN1-CT interacted with the full-length and the MH2 domain of XSmad1, but not with its MH1 domain, indicating that the MH2 domain mediates the association of XMAN1 and XSmad1. GST-XMAN1-CT also strongly associated with BMP-responsive Smads, Smad5 and Smad8, weakly with Smad2, but not with XSmad4α and inhibitory Smads, Smad6 and Smad7. These results suggest that XMAN1 preferentially associated with BMP-responsive Smads in vitro.

XMAN1 activity in neural development

To analyze the role of endogenous XMAN1, we suppressed XMAN1 activity by injecting antisense morpholino oligos (XMAN1-MO) complementary to the sequence around the initiation codon. As controls, we injected nonspecific control morpholino (CTL-MO) and four-base-mismatched morpholino oligos (XMAN1-4mmMO). We first examined the specificity of XMAN1-MO using an XMAN1 construct in which the 5′UTR is retained to hybridize with XMAN1-MO and which encodes Myc-tags at the C terminus (referred to as 5′UTR-XMAN1-MT). Co-injection of XMAN1-MO inhibited the translation of 5′UTR-XMAN1-MT mRNA at the early gastrula stage (stage 10.5), but co-injection of XMAN1-4mmMO did not (Fig. 8A). This inhibitory effect of XMAN1-MO on the translation of 5′UTR-XMAN1-MT mRNA was also observed at the tailbud stage (stage 25). The specificity of XMAN-MO was further examined by real-time PCR with animal caps. Injection of 5′UTR-XMAN1-MT mRNA induced *nrp1* expression in animal caps as does MT-XMAN1 mRNA that lacks the annealing sequence for XMAN1-MO (Fig. 8B). Co-injection of XMAN1-MO suppressed the expression of *nrp1* induced by 5′UTR-XMAN1-MT mRNA but not by MT-XMAN1 mRNA, indicating that XMAN1-MO specifically suppresses the neuralizing activity of 5′UTR-XMAN1-MT.

We next investigated the effects of XMAN1-MO on early *Xenopus* embryogenesis. Although XMAN1-MO-injected embryos (XMAN1 morphants) gastrulated normally in the same way as uninjected and 4mmMO-injected embryos, the effects of XMAN1-MO became evident at later stages. When 4mmMO was injected into the right sides of embryos, morphological anomalies were not observed (100%, *n*=48, two experiments; Fig. 8C, parts b,f). By contrast, the right eyes of XMAN1 morphants were absent or poorly formed (96%, *n*=62, three experiments; Fig. 8C, parts 8,9). This eye-less phenotype was completely or partially rescued by co-injection with MT-XMAN1 mRNA (14% and 57%, respectively, *n*=90, two experiments; Fig. 8C, parts d,f).

Because XMAN1 antagonizes BMP signaling as mentioned

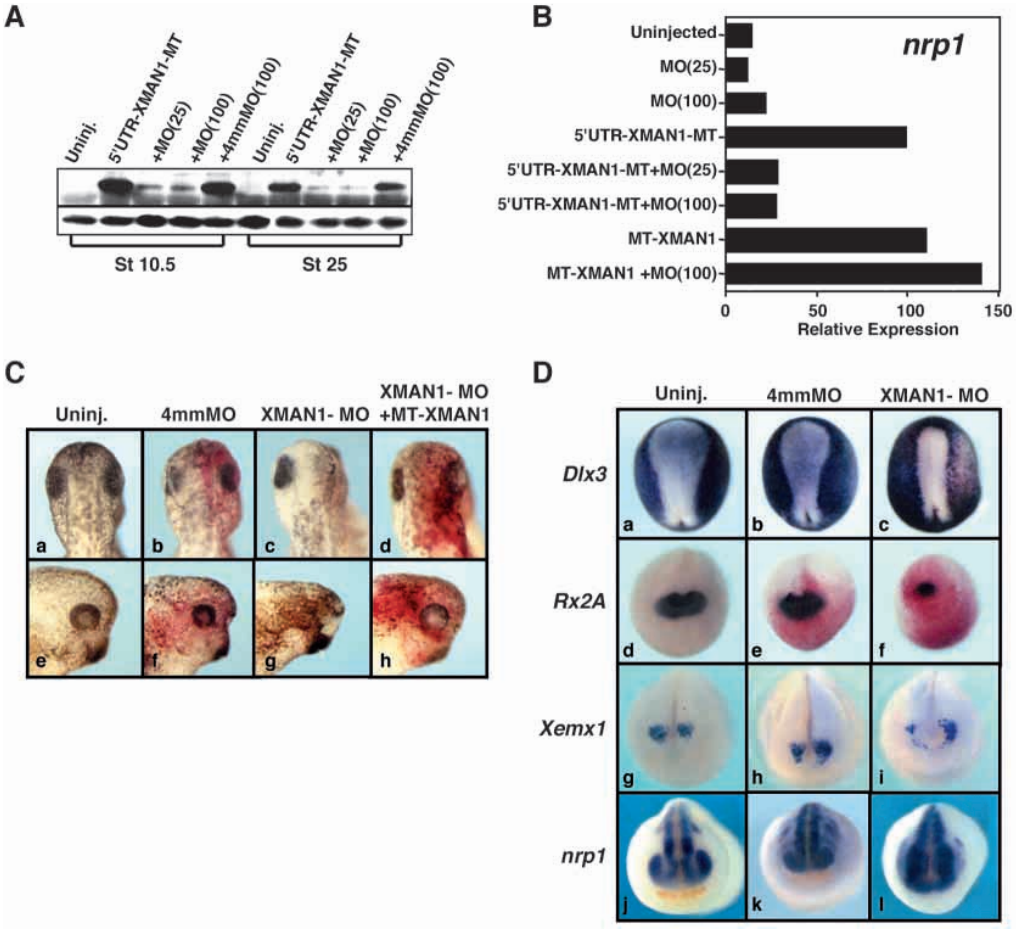

Fig. 8. Roles of XMAN1 during *Xenopus* development. (A) XMAN1 antisense morpholino oligo (XMAN1-MO) inhibits the translation from synthetic *XMAN1* mRNA. Extracts prepared from stage 10.5 and stage 25 embryos injected with Myc-tagged *XMAN1* mRNA possessing the XMAN1-MO annealing sequence (5′UTR-XMAN1-MT) together with XMAN1-MO or XMAN1-4mmMO (doses in ng per embryo are in parentheses) were subjected to immunoblotting with anti-Myc antibody (upper panel) and with anti-β-tubulin antibody (lower panel). (B) XMAN1-MO suppresses neural induction by XMAN1. Real-time RT-PCR analysis for *nrp1* with animal caps at the equivalent of stage 25. Doses of injected mRNA, 500 pg per embryos. Doses of XMAN1-MO are indicated in parentheses (ng per embryo). (C) Morphological appearance of injected embryos. XMAN1-4mmMO (b,f), XMAN1-MO (c,g) and XMAN1-MO plus MT-XMAN1 mRNA (d,h) were injected together with β-gal mRNA as a tracer (b-d,f-h) into two right animal blastomeres at the four-cell stage. XMAN1-MO injection caused a reduced eye on the injected side (c,g), which is rescued by co-injection of MT-XMAN1 mRNA (d,h). Injected doses (per blastomere): MOs, 25 ng; MT-XMAN1 mRNA, 67.5 pg; β-gal mRNA, 15 pg. a-d, dorsal view (anterior is upwards); e-h, lateral view of a-d, respectively, showing the right side of the embryos. (D) Whole-mount in situ analysis for *Dlx3* (epidermis, stage 13), *Rx2A* (eye, stage 20), *Xemx1* (telencephalon, stage 20) and *nrp1* (pan-neural, stage 20) expression in uninjected, XMAN1-4mmMO- and XMAN1-MO-injected embryos as indicated. a-c, dorsal view (anterior is upwards); d-l, anterior view (dorsal is upwards). β-Gal mRNA was co-injected in b,c,e,f.

above, suppression of XMAN1 activity by XMAN1-MO might lead to upregulation of BMP activity and hence causes eye defects as shown above. To address this possibility, we examined the expression of Dlx3, an epidermal marker (Feledy et al., 1999), in XMAN1 morphants. As shown in Fig. 8D, the Dlx3-negative region corresponding to the future neural plate was shortened and narrowed particularly in the anterior region at the early neurula stage 13 (n=18/20). The expression domain of *Msx1*, another epidermal marker and a direct target of BMP signaling (Suzuki et al., 1997), was also expanded in XMAN-morphants (n=8/8, not shown). In addition, expression domains of *Rx2A* (retina) (Mathers et al., 1997), *Xemx1* (dorsal telencephalon) (Pannese et al., 1998), *nrp1* (Knecht et al., 1995) and *Xsix3* (forebrain) (Zhou et al., 2000) were severely reduced (Fig. 8Ci; *Rx2A*, n=18/20; *Xemx1*, n=4/6; *nrp1*, n=8/9; *Xsix3*, n=10/10, not shown). When XMAN1-MO was injected into all animal blastomeres at the four-cell stage, development of anterior structures was also severely reduced. Furthermore, when XMAN1-MO was injected dorsally at the four-cell stage, similar phenotypes were observed but expression of dorsal markers, *goosecoid* and *chordin*, was not affected (not shown). These data imply that the XMAN1-MO phenotype is caused by a decrease in XMAN1 activity in the anterior neuroectoderm where XMAN1 is expressed, and not by a reduction in the dorsal mesoderm. Taken together, these results suggest that the XMAN1 function is required for anterior neural development.

DISCUSSION

XMAN1 is a novel modifier for BMP signaling

Inner nuclear membrane proteins have been mainly implicated in disassembly and reassembly of the nuclear envelope during mitosis, based on their proximity to the nuclear lamina and chromatin (Worman and Courvalin, 2000). In this study, we have shown that the inner nuclear membrane protein XMAN1 antagonizes the BMP pathway. Suppression of BMP downstream target genes (*Xhox3*, *Xvent1*, *Xvnet2* and *Msx1*) by XMAN1 was observed in our assays using animal caps, ventral marginal zones and a luciferase reporter, supporting the idea that XMAN1 can behave as an inhibitor for BMP signaling when overexpressed. This wide range of inhibitory effects of XMAN1 on BMP target genes suggests that XMAN1

negatively regulates a variety of biological processes mediated by the BMP pathway.

We presume that the inhibitory action of XMAN1 to BMP signaling is due to its binding specificity with the BMP-responsive Smads. XSmad1 interacts with XMAN1 via its MH2 domain, to which several transcription factors, co-activators and co-repressors bind (Massague and Chen, 2000; Wrana, 2000). Therefore, XMAN1 may compete with those transcription factors or coactivators for binding to the MH2 domain, or it may recruit a co-repressor on the MH2-domain as has been suggested for the Ski oncoprotein (Wang et al., 2000). To address these questions it would be helpful to find other binding proteins to the XMAN1/Smad1 (or Smad5, Smad8) complex.

The presence of the LEM domain in XMAN1 implies that XMAN1 on the inner nuclear membrane interacts with BAFs through the LEM domain and thus affects DNA indirectly. However, the role of the LEM domain in inhibiting BMP signaling is still unclear, because deletion of the LEM domain did not affect the activity of XMAN1 in neuralizing ectoderm (Fig. 4C).

Interactions between XMAN1 and XSmad1

Strong structural similarity of XMAN1 to human MAN1 enabled us to deduce that XMAN1 localizes in the inner nuclear membrane. This is supported by confocal immunofluorescent microscopy in which the subcellular localization of XMAN1 is very similar to that of emerin, a known inner nuclear membrane protein (Manilal et al., 1996; Nagano et al., 1996). Given that XMAN1 localizes to the inner nuclear membrane, how does it regulate BMP signaling? According to a current model, the C-terminal region of human MAN1 is likely to face the nucleoplasm (Lin et al., 2000). Thus, in the simplest way, activated Smad1 or Smad1/co-factor/transcription complex in the nucleus may be trapped and targeted to the inner nuclear membrane through binding to the C-terminal region of XMAN1, leading to disruption of the transcription complex on BMP target genes. Actually, several transcription factors have been shown to become inactive when they are localized to the nuclear envelope (Cohen et al., 2001). However, XMAN1-CT, which lacks the transmembrane domains necessary for its nuclear membrane localization (Wu et al., 2002) and is ubiquitously distributed within the cell (Fig. 4D), still showed neuralizing activity (Fig. 4C). This implies that the physical interaction between XSmad1 and XMAN1-CT irrelevant to subcellular localization is enough to antagonize the BMP pathway under our experimental conditions. Nevertheless, we have noticed that full-length XMAN1 reproducibly shows stronger activity than XMAN1-CT in suppressing the expression of BMP downstream targets in animal caps and *Xvent2*-Luc activation (Fig. 6E), suggesting that some domains outside the C-terminal region or nuclear localization of XMAN1 are necessary for the full activity.

In the case of the TGF-β/activin-responsive Smads, Smad2 and Smad3, as well as Smad4, it has recently been shown that nucleocytoplasmic shuttling of those Smad proteins occurs constitutively (Inman et al., 2002; Xu et al., 2002), and that this process for Smad2 is conducted by the nucleoporins, CAN/Nup214 and Nup153 (Xu et al., 2002). The balance of those Smads between the cytoplasm and the nucleus is thought be maintained by the cytoplasmic retention factors (SARA, microtubules and the actin binding protein filamin) and Smad-interacting transcription factors in the nucleus, such as FAST-1 (Tsukazaki et al., 1998; Dong et al., 2000; Sasaki et al., 2001; Xu et al., 2002). Phosphorylation of Smad2 shifts the balance to increase the nuclear pool of Smad2. However, it has not been clarified whether BMP-responsive Smads are regulated by similar mechanisms, while both the nuclear localization signal (NLS) and the nuclear export signal (NES) have been identified in Smad1 (Xiao et al., 2001). The association of XMAN1 and Smad1/Smad5/Smad8 may regulate the amount of Smads accessible to downstream target genes at the nuclear membrane level.

RRM in XMAN1

We found an RRM in the C-terminal region of XMAN1 that had not been described before. Interestingly, this RRM is required for the neuralizing activity of XMAN1 (Fig. 4C). Because RRMs are frequently found in RNA-binding proteins and mediate their association with RNAs (Birney et al., 1993), it might be possible that RNAs mediate the neuralizing activity of XMAN1. However, in our immunoprecipitation experiments, RNase treatment of the cell lysate did not abolish the interaction of XMAN1-CT and XSmad1 (data not shown). In addition, GST pull-down assays show that XMAN1 and BMP-responsive Smads can interact directly in vitro (Fig. 7C). These results suggest that the association between the two proteins is mediated by protein/protein interaction rather than via RNA, although we cannot exclude the possibility that some RNA may interact with XMAN1 to modify BMP signaling or to exert an unidentified role. RRM functions as a protein/protein interaction domain as exemplified by PTB-associated splicing factor (Dye and Patton, 2001). Involvement of the RRM in binding to Smads has not been reported before. We could not find any sequence similarity between the RRM of XMAN1 and known Smad-interacting motifs found in some Smad-binding proteins, such as OAZ, Smurf1, FAST1 and Mixer, and phenylalanine-glycine (FG) repeats required for Smad-binding in some nucleoporins (Massague and Chen, 2000; Wrana, 2000; Moustakas et al., 2001; Randall et al., 2002; Xu et al., 2002).

Role of XMAN1 in early embryogenesis

Interference with endogenous XMAN1 functions by morpholino oligos produced defects in anterior development (Fig. 8). The affected regions, eyes and anterior CNS, correspond well to the expression domains of XMAN1 at the neurula to tailbud stages (Figs 3, 8). Upregulation of the epidermalizing activity of BMP signaling in XMAN1 morphants seems to be responsible for the XMAN1-MO phenotype. The expression domain of Dlx3, an epidermal marker, was expanded in XMAN1 morphants, and concomitantly, the future neural plate was shortened and narrowed, particularly in the anterior region where XMAN1 is normally expressed at the neurula stage. By contrast, in XMAN1-MO-injected embryos, the expression of Sox2, a downstream target of chordin and one of the earliest markers of the neuroectoderm (Mizuseki et al., 1998a), was not greatly affected at the gastrula stage (not shown). Because XMAN1 induces neural tissues in animal caps independent of Chordin (Fig. 6B), and dorsal injections of XMAN1-MO do not affect the expression of *chordin* (not shown), Sox2 expression may

be regulated in an XMAN1-independent manner, or by maternal XMAN1. It has been shown that translation from maternal mRNAs is not efficiently inhibited by antisense morpholinos (Heasman et al., 2000). Considering the fact that *XMAN1* is initially expressed in the entire ectoderm prior to gastrulation (Fig. 3B), maternal XMAN1 may sensitize the ectoderm to neural induction by attenuating BMP signaling prior to organizer-derived BMP antagonists (Fig. 6C).

Augmentation of BMP signaling by XMAN1-MO would affect other BMP-dependent biological processes. We found that the development of XMAN1 morphants into which XMAN1-MO was injected radially into the animal region was slower than that of wild type after tailbud stages. Their development finally ceased and they began to die. Because the TAK1-TAB1 pathway is activated in a BMP-dependent manner and is involved in cell death (Shibuya et al., 1998), it should be interesting to investigate whether XMAN1 regulates the cell death initiated by BMP the signaling.

Recently, it has been shown that the epithelial and sensorial layers of the ectoderm have different competences to neuronal-promoting factors, and these competences are established before gastrulation (Chalmers et al., 2002). In this sense, it should be noted that XMAN1 is expressed mainly in the sensorial layer of the ectoderm at late blastula to neurula stages, where primary neurons are generated. XMAN1 may be involved in generating the difference between the two layers of neuroectoderm, perhaps establishing competence to neurogenesis by modulating BMP signaling.

In this paper, we have presented the first evidence that an inner nuclear membrane protein is likely to have a role in signal transduction pathways. It will be interesting to analyze how XMAN1 functions as a modulator in BMP signaling during embryogenesis as well as in adulthood, when an autoimmune disease – in which one of the autoantibody-reacting epitopes is MAN1 – is known to occur in humans.

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