XPACE4 is a localized pro-protein convertase required for mesoderm induction and the cleavage of specific TGFβ proteins in *Xenopus* development

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Summary

XPACE4 is a member of the subtilisin/kexin family of pro-protein convertases. It cleaves many pro-proteins to release their active proteins, including members of the TGFβ family of signaling molecules. Studies in mouse suggest it may have important roles in regulating embryonic tissue specification. Here, we examine the role of XPACE4 in *Xenopus* development and make three novel observations: first, *XPACE4* is stored as maternal mRNA localized to the mitochondrial cloud and vegetal hemisphere of the oocyte; second, it is required for the endogenous mesoderm inducing activity of vegetal cells before gastrulation; and third, it has substrate-specific activity, cleaving Xnr1, Xnr2, Xnr3 and Vg1, but not Xnr5, Derrière or ActivinB pro-proteins. We conclude that maternal XPACE4 plays an important role in embryonic patterning by regulating the production of a subset of active mature TGFβ proteins in specific sites.

Key words: PACE4, TGFβ, Mesoderm induction, Vg1

Introduction

In *Xenopus*, the initial specification of the mesoderm and endoderm germ layers relies on the coordinated activities of stored maternal regulators; the T-box transcriptional activator VegT and the derepressor β-catenin (Kofron et al., 1999; Xanthos et al., 2002; Xanthos et al., 2001; Zhang et al., 1998). These directly regulate the expression of zygotic transcription factors and signaling molecules, which in turn establish the endoderm and induce the neighboring mesoderm. With the onset of zygotic transcription in *Xenopus*, the signaling milieu around the embryonic cells rapidly becomes complex. VegT and β-catenin initiate the expression of at least seven TGFβ family members (nodal-related proteins 1-6, and *derrière*) and three FGFs (*Fgf3*, *Fgf4* and *Fgf8*). Other TGFβ family members, Vg1 and BMP2, are inherited maternally, and *Bmp4* and *Bmp7* are expressed zygotically soon after the mid-blastula transition. Here, we examine the role in this process of the pro-protein convertase, XPACE4 (paired basic amino acid converting enzyme 4)/SPC4 (subtilisin-kexin like pro-protein convertase 4).

XPACE4 is a secreted, heparin-binding member of the pro-protein convertase (PC) family (Mains et al., 1997; Tsuji et al., 2003). It recognizes the multibasic consensus motif, RXXR, and cleaves substrates, including TGFβ family members, to release the active mature protein. PCs are important in vertebrate embryogenesis (for a review, see Taylor et al., 2003). In vertebrate embryos, they have been shown to process activin (Roebroek et al., 1993), TGFβ1 (Dubois et al., 1995), BMPs (Cui et al., 1998) and Nodal (Constam and Robertson, 1999; Beck et al., 2002; Constam and Robertson, 2000). PCs have also been implicated in remodeling of the extracellular matrix by processing matrix metalloproteases (Leighton and Kadler, 2003; Yana and Weiss, 2000) and regulating cell adhesion by processing integrins (Berthet et al., 2000; Stawowy et al., 2004). Recently glypican 3, a heparan sulfate proteoglycan involved in morphogen gradient formation was found to be processed by PCs (De Cat et al., 2003).

In this study, we have analyzed the role of XPACE4 in mes-endoderm specification in *Xenopus*. We find that *XPACE4* maternal mRNA is localized to the vegetal hemisphere of oocytes, whereas zygotic mRNA is localized to the notochord, the brain and a subset of endodermal precursors. We show that XPACE4 protein is essential for normal development in vivo, and for the production of endogenous mesoderm inducing signals. By comparing the cleavage of overexpressed tagged TGFβ proteins in wild-type and *XPACE4*-depleted backgrounds, we identify the TGFβ proteins that are specific substrates for cleavage by XPACE4.

Materials and methods

Cloning of *XPACE4*

Degenerate oligonucleotides were designed based on sequence motifs conserved among multiple members of the PC family. A partial length *XPACE4* cDNA was amplified from oocyte cDNA by PCR using the oligonucleotide pair downward [5′-TGG GG(A/T/C) CC(A/T) GA(A/T/C/G) GA(T/C) GA(T/C) GG-3′ (coding for WGP(D/E)DG)] and upward [5′-AC(A/T) TGG (A/C)G(A/G/T) GA(T/C) (A/G)T(G/T) CA(G/A)-3′ (coding for TWRC(M/V)QH)]. This cDNA was used to screen a *Xenopus* oocyte library and two partial-length *XPACE4* clones were obtained and fully sequenced. Both of these lacked sequence encoding N- and C-terminal regions of the protein based on alignment with PACE4 from other species. cDNAs encoding a single XPACE4 N terminus and two distinct C termini were obtained using 5′ and 3′ Rapid Extension of cDNA Ends (RACE), respectively (AY836768 and AY836769). A single cDNA containing the entire open reading frame of the longer of the two *XPACE4* splice isoforms was amplified from oocyte cDNA using PCR and was used for all subsequent studies.

Oligos and mRNAs

The antisense oligonucleotide complementary to *XPACE4* used in this study (AS-5) was 5′-C*A*A*GGTTCAGGTAGCC*G*T*-3′, where residues with phosphorothioate bonds are indicated by an asterisk (*) and was used in doses of 4-5 ng. *XPACE4* morpholino oligo was 5′-GCATGTTTGAAATGCTCAGAGGGAG-3′ and was used in doses of 30-45 ng.

To generate HA-tagged TGFβs, one HA epitope (YPYDVPDA) was inserted at the C terminus immediately after the coding sequence, and before the stop codon by high fidelity PCR. The inserts were subcloned into pCS2+ vector. Each construct was then sequenced for confirmation and tested for activity to ensure that the activity of the protein was not disrupted by the presence of the tag. The linearized plasmids were purified and mRNA was transcribed with SP6 polymerase using the Megascript kit (Ambion). The doses and sites of injection are described in the text.

Real-time PCR

Total RNA and cDNA were prepared according to Zhang et al. (Zhang et al., 1998). cDNA was synthesized using oligo dT primers, or random hexamer (R6) primers where indicated. Real-time RT-PCR was carried out using the Light Cycler System (Roche) as described by Kofron et al. (Kofron et al., 2001) using the primers and cycling conditions as listed in Table 1. All samples were normalized to levels of ornithine decarboxylase (*ODC*), which was used as the loading control. Every experiment was repeated at least twice with different oocyte and embryo batches to show that the results were reproducible.

Table 1. PCR primers and cycling conditions

Primers	Origin	Sequence	Denaturing temp	Annealing temperature (°C)/time (seconds)	Extension temperature (°C)/time (seconds)	Acquisition temperature (°C)/time (seconds)
Cardiac actin	XMMR*	U: 5-TCC CTG TAC GCT TCT GGT CGT A –3 D: 5-TCT CAA AGT CCA AAG CCA CAT A-3	95	58/5	72/12	83/3
Chordin	XMMR*	U: 5-AAC TGC CAG GAC TGG ATG GT-3 D: 5-GGC AGG ATT TAG AGT TGC TTC-3	95	55/5	72/12	81/3
Derrière	Kofron et al., 1999	U: 5-TGG CAG AGT TGT GGC TAT CA-3 D: 5-CTA TGG CTG CTA TGG TTC CTT-3	95	55/5	72/18	82/3
FGF8	Kofron et al., 1999	U: 5-CTG GTG ACC GAC CAA CTA AG-3 D: 5-ACC AGC CTT CGT ACT TGA CA-3	95	55/5	72/14	86/3
Furin	New	U: 5- TGTAGCCTCCAGCCAGTCTT-3 D: 5- GGCAGTCATTGATTGTGGTG-3	95	58/5	72/9	82/3
Goosecoid v.2	Kofron et al. 2004	U: 5′-TTC ACC GAT GAA CAA CTG GA-3′ D: 5′-TTC CAC TTT TGG GCA TTT TC-3′	95	55/5	72/11	82/3
MyoDa	XMMR*	U: 5-AGC TCC AAC TGC TCC GAC GGC ATG AA-3 D: 5-AGG AGA GAA TCC AGT TGA TGG AAA CA-3	95	55/5	72/18	86/3
ODC	Heasman et al., 2000	U: 5-GCC ATT GTG AAG ACT CTC TCC ATT C-3 D: 5-TTC GGG TGA TTC CTT GCC AC-3	95	55/5	72/12	83/3
XPACE4	New	U: 5- TGTCTCCTAGCGGGATGACT-3 D: 5- CTGGCGCAGAAAATGTAGGT-3	95	55/5	72/10	84/3
N-CAM	Yokota et al., 2003	U: 5-CAC AGT TCC ACC AAA TGC-3 D: 5-GGA ATC AAG CGG TAC AGA –3	95	60/5	72/13	84/3
Vg1	New	U: 5- CCATACCCGCTGACAGAAAT-3 D: 5- CCTGCAGCCACACTCATCTA-3	95	58/5	72/8	80/3
Xbra	Sun et al., 1999	U: 5-TTC TGA AGG TGA GCA TGT CG-3 D: 5-GTT TGA CTT TGC TAA AAG AGA CAG G-3	95	55/5	72/8	75/3
Xnr1	Kofron et al., 1999	U: 5-TGG CCA GAT AGA GTA GAG-3 D: 5-TCC AAC GGT TCT CAC TTT-3	95	55/5	72/12	81/3
Xnr-3	Kofron et al., 1999	U: 5-CTT CTG CAC TAG ATT CTG-3 D: 5-CAG CTT CTG GCC AAG ACT-3	95	57/5	72/10	79/3
XSox17α	Xanthos et. al. 2001	U: 5-GCA AGA TGC TTG GCA AGT CG-3 D: 5-GCT GAA GTT CTC TAG ACA CA-3	95	58/5	72/8	85/3

**Xenopus* molecular marker resource http://www.xenbase.org/xmmr/Marker_pages/primers.html

Oocytes and embryos

Full-grown oocytes were isolated and cultured as described previously (Xanthos et al., 2001), before being injected with antisense oligos and fertilized using the host transfer technique as described previously (Zuck, 1998). Staging was according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1967).

Whole-mount in situ hybridization

Oocytes at all stages were removed from follicle cells by incubating in 2 mg/ml collagenase in Ca^{2+} and Mg^{2+}-free 1×MMR and fixed in MEMFA for 1-2 hours. To increase probe penetration into the yolky vegetal hemisphere, fixed oocytes and embryos were bisected into halves in the animal-vegetal axis using a clean scalpel blade, refixed in MEMFA, washed in PBS and transferred into 100% methanol. Whole-mount in situ protocol was modified from Harland (Harland, 1991). *XPACE4* antisense probe was transcribed using T7 polymerase after pCS2+*XPACE4* vector was digested with *Eco*RI. For the sense probe, pCS2+ *XPACE4* vector was digested with *Xho*I and transcribed using SP6 polymerase. In situs were developed using BM Purple as substrate (Roche).

Nieuwkoop assay

Vegetal masses were dissected from control and *XPACE4*-depleted embryos at stage 9. Wild-type animal caps from stage 8-9 embryos were co-cultured with vegetal masses for 1-2 hours. They were then separated carefully using tungsten needles and contaminating vegetal cells, recognized by their vital dye coloring, were removed. The caps were incubated until they reached the desired stage and were frozen down for analysis.

Paracrine assay

The paracrine assay was carried out as described previously (Lustig and Kirschner, 1995). Oocytes were injected with an antisense oligo, cultured for 48 hours at 18°C, and then injected vegetally with 150-200 pg of *Xnr1* mRNA, before culturing for an additional 12-24 hours to allow time for protein synthesis and processing. The oocytes were transferred into wells in agar plates with indentations, and stage 9 animal caps were placed on their animal poles, bringing the inner cell layer of caps into contact with the surface of the oocytes. Caps were co-cultured with oocytes in OCM for 1 hour at room temperature, and then carefully separated from the oocytes. The caps were cultured separately until sibling embryos reached the desired stage when they were frozen down for analysis.

Luciferase assay

Control and *XPACE4*-depleted embryos were injected vegetally at the two-cell stage with 50 pg of the firefly luciferase reporter construct pGL3-ARE-luciferase described previously (Huang et al., 1995) together with 20 pg of control HSTK *Renilla luciferase* plasmid. Batches of four embryos were collected in triplicate for each injection mixture at stage 10.5. Luciferase assays were performed using the Dual Luciferase Reporter Assay system (Promega) as described (Kofron et al., 2004). The assay was repeated twice, and a representative experiment is shown.

Blastocoel fluid withdrawal

Needles were prepared in the size range of 10-20 nl/second, and rinsed with the protease inhibitor PIC (Roche). The needle was inserted through the blastocoel roof into the blastocoel cavity of early gastrula stage embryos, and the blastocoel fluid was withdrawn gradually, using a Medical Systems picoinjector. Care was taken not to contaminate the fluid with cellular debris. Depending on the batch of embryos, 0.1-0.2 μl of fluid/embryo was collected. After blastocoel fluid from 10 embryos was collected, the fluid was snap frozen. Similar volumes of fluid were withdrawn from control and experimental embryos.

Western blots

Protein extracts were prepared from batches of 3-5 embryos using 10 μl homogenization buffer/embryo. Homogenization buffer for anti-HA blots is 2.5% IGEPAL CA-630 Sigma I3021, 50 mM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% SDS; containing 1:100 PIC (Sigma P-8340). For anti-phospho-SMAD2 blots, homogenization buffer is 20 mM Tris pH 8.0, 2 mM EDTA, 5 mM EGTA, 0.5% NP-40 (Sigma), 25 mM sodium β-glycerophosphate, 100 mM NaF, 20 nM Calyculin A, 10 mM sodium pyrophosphate, containing 1:100 PIC and PMSF. Homogenates were spun at 13,000 ***g*** for 10 minutes and the supernatant was collected and mixed with equal volume of 2× reducing sample buffer. Blastocoel fluid samples were also spun down and 10 μl of sample buffer was added. Samples were boiled and 0.5-1 embryo equivalents of protein extracts or the entire blastocoel fluid samples were loaded on 10-12% SDS-PAGE gels and transferred on to nitrocellulose membranes. The membranes were blocked with 5% NFDM in PBS-Tween overnight at 4°C for anti-phospho-Smad2 blots and 1-2 hours at room temperature for others. Anti-phospho-Smad2 antibody (Cell Signaling Technology #3101) and anti-Smad2 antibody (BD Transduction Laboratories) were used at a dilution of 1:500. Anti-HA high affinity rat monoclonal antibody 3F10 (Roche 1-867-423) was used at a dilution of 1:1000. Signal detection was carried out using the Amersham ECL detection system. No signal was detected with this HA antibody in the uninjected control samples. The relative mobility of each protein was calculated by comparison with a standard curve generated by migration of the prestained kaleidoscope standards (BioRad). One HA tag is approximately 1.1 kDa.

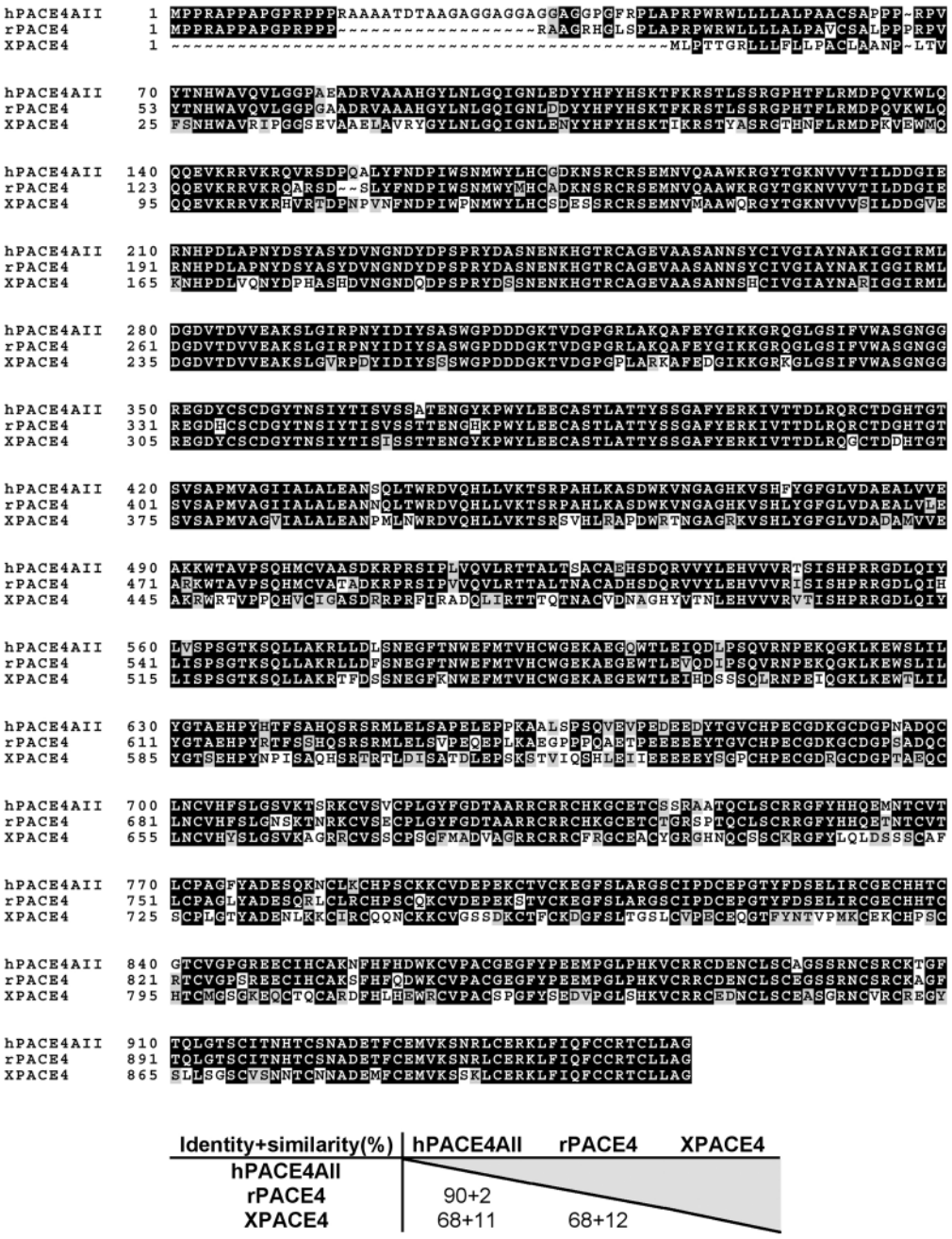
As a loading control, membranes were stripped after signal detection, and incubated with α-tubulin antibody (DM1A, Neomarkers) at 1:10,000. No α-tubulin was detected in the blastocoel fluid samples. The experiments were repeated at least twice.

Results

Xenopus PACE4 is a vegetally localized maternal mRNA

The predicted amino acid sequence of one splice isoform of XPACE4 is shown in Fig. 1. It is most closely related to rat PACE4 (rPACE4) and human PACE4A-II isoform (hPACE4A-II). To determine the temporal expression profile of *XPACE4* mRNA, real-time RT-PCR was performed using *XPACE4*-specific primers on a staged series of embryos. Fig. 2A shows that *XPACE4* mRNA is present in oocytes and continues to be expressed throughout blastula and gastrula stages with maximum expression at the early blastula stage. As mRNA levels increase before mid-blastula transition (MBT), when transcription is considered to be absent, we asked whether this was due to polyadenylation. We compared oligo dT primed versus random hexamer primed cDNA for *XPACE4* levels. Fig. 2B shows that *XPACE4* mRNA levels remain constant at early blastula stage compared with oocyte levels in random hexamer primed cDNA, indicating that the increase in *XPACE4* levels is due to polyadenylation of the maternal mRNA. Northern blot analysis reveals the presence of three distinct *XPACE4* RNA species that range in size from 2.5 to 7 kb and show differing patterns of temporal regulation (data not shown).

To determine the spatial localization of *XPACE4* mRNA during oogenesis, real-time RT-PCR was performed using oocytes dissected into animal-vegetal halves, and embryos dissected into dorsal and ventral halves or equatorial and vegetal explants. *XPACE*4 mRNA is enriched in vegetal halves of oocytes and vegetal masses of embryos, with no significant



Identity+similarity(%)	hPACE4AII	rPACE4	XPACE4
hPACE4AII			
rPACE4	90+2		
XPACE4	68+11	68+12	

Fig. 1. Alignment of *Xenopus* PACE4 (XPACE4) protein sequence with human PACE4 splice isoform AII (hPACE4AII) and rat PACE4 (rPACE4). The clustal alignment algorithm of MacVector software is used to align the translated protein sequences and BOXSHADE is used for display. The table shows the percent identity and similarity of these proteins. *XPACE4* is cloned as described in the Materials and methods procedures. Accession Numbers: hPACE4AII, NP 612192; rPACE4, NP 037131; XPACE4, AY836768.

asymmetrical distribution between dorsal and ventral halves (data not shown). To confirm the localized expression pattern of *XPACE4*, we performed whole-mount in situ hybridization using *XPACE4* antisense probe at different stages of oogenesis and embryogenesis. Fig. 2C shows that in stage 1 oocytes, *XPACE4* localizes to the mitochondrial cloud. As the mitochondrial cloud disperses at stage 2 (Heasman et al., 1984), *XPACE4* mRNA co-localizes with the vegetal cortex where it remains for the rest of oogenesis (Fig. 2C; inset). Hemi-sections of full-grown oocytes reveals that *XPACE4* mRNA is not exclusively associated with the cortex of the vegetal hemisphere but is also diffusely distributed in the vegetal cytoplasm and excluded from the animal hemisphere (Fig. 2D). Fig. 2E,F shows that at the gastrula stages, *XPACE4* mRNA persists vegetally and becomes localized to the presumptive endoderm. After gastrulation, *XPACE4* expression declines dramatically and Fig. 2G shows that it is restricted to a small number of cells in the endoderm. Fig. 2H shows that at tailbud stages, the signal is detected specifically in the olfactory bulb, the brain and in the notochord (inset shows staining in the notochord in a transverse section with sense control on the right).

These results show that maternal *XPACE4* mRNA is localized to the vegetal hemisphere of the oocyte and is regulated by polyadenylation, while during embryogenesis, it is localized in endodermal precursors and later in specific areas of all three germ layers.

Depletion of maternal *XPACE4* mRNA results in reduced enzymatic function in paracrine assays

Antisense oligos designed against the *XPACE4* coding sequence were tested for efficiency by injection into the vegetal poles of full-grown oocytes, which were incubated for 48 hours before real-time RT PCR analysis for *XPACE4* mRNA depletion. Fig. 3A shows that antisense oligo 5 (AS-5) is the most efficient, and depletes *XPACE4* mRNA to 5-10% of control levels. To increase the stability of oligo, phosphorothioate modified AS-5 (AS-5MP) was used. Fig. 3B shows that the oligo does not affect mRNA levels of a related maternal convertase, furin (*XFurA*).

To determine when zygotic *XPACE4* mRNA is first expressed in maternal *XPACE4*-depleted embryos, we assayed a series of developmental stages comparing *XPACE4*-depleted

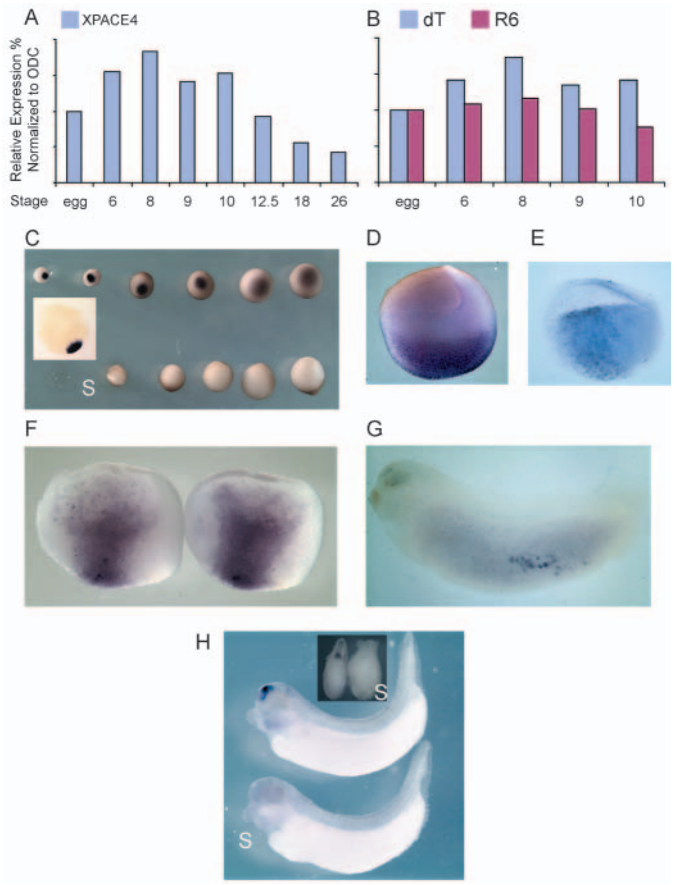


Fig. 2. Spatiotemporal expression pattern of *XPACE4*. (A) Real-time RT-PCR analysis of the wild-type embryos at the indicated stages shows that *XPACE4* mRNA is abundant maternally and its level increases pre-MBT (stage 6 and 8) and gradually declines after gastrulation (stage 10 and 12.5). (B) The comparison of oligo dT (dT) versus random hexamer (R6) primed cDNA shows the increase in *XPACE4* levels pre-MBT is due to polyadenylation of the maternal mRNA. (C) Whole-mount in situ hybridization shows that XPACE4 is localized during early oogenesis. Inset shows *XPACE4* mRNA localization in the mitochondrial cloud of stage 1 oocytes. No signal is detected with the sense probe (S). (D) *XPACE4* is localized to the vegetal hemisphere of full-grown oocytes. (E,F) Hemisected stage 10 (E) and stage 11 (F) embryos show *XPACE4* mRNA remains localized to the endodermal precursors during gastrulation (dorsal side on the right). (G) At stage 30, *XPACE4* is detected in a group of cells in the endoderm after the embryos are bleached and cleared. (H) At stage 35/36, *XPACE4* is detected in the olfactory bulb, the brain and the notochord. The lower embryo is the sense control. Inset shows the notochord in transverse sections. S, sense control.

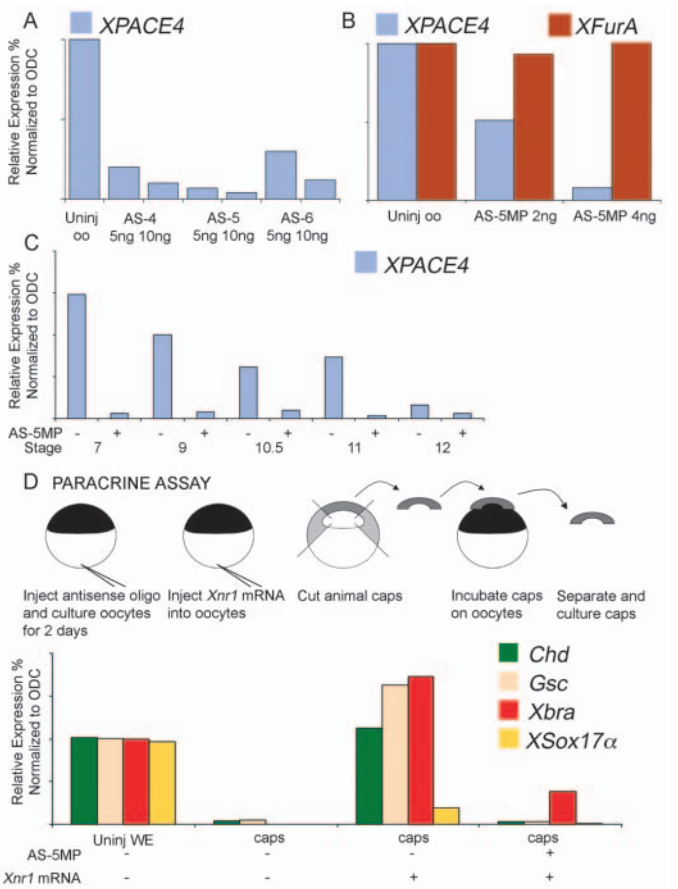


Fig. 3. Depletion of *XPACE4* using an antisense oligo approach. (A) Real-time RT-PCR analysis of oocytes (oo) injected with 5-10 ng unmodified antisense oligos shows depletion of maternal *XPACE4* mRNA to variable degrees. (B) Phosphorothioate modified AS-5 oligo (AS-5MP) depletes *XPACE4* mRNA without affecting the mRNA levels of a closely related maternal convertase Furin (*XFurA*) as analyzed by real-time RT-PCR. (C) *XPACE4* mRNA levels in control and *XPACE4*-depleted embryos: *XPACE4*-depleted embryos (AS-5MP+) are generated by the host transfer technique and are compared with sibling uninjected control embryos at blastula (7 and 8) and gastrula (10, 11, 12) stages. Real-time RT-PCR shows that *XPACE4* is depleted down to 5% and it does not reach the control levels (AS-5MP–). (D) Antisense oligo depletion of *XPACE4* reduces signaling activity of Xnr1 in oocytes. A schematic presentation of the paracrine assay is shown at the top. Animal caps co-cultured with uninjected control oocytes have very low or no detectable levels of organizer genes *chordin* (*Chd*), *goosecoid* (*Gsc*), mesodermal gene *Xbra* or endodermal gene *XSox17α*. All of these genes are induced when caps are co-cultured with control oocytes injected with *Xnr1* mRNA. Animal caps co-cultured with *XPACE4*-depleted oocytes injected with *Xnr1* mRNA show a reduction in the expression levels of these mRNAs. Sibling whole embryo (WE) is used for the dilution series and the quantification in real-time RT-PCR.

embryos with controls. Fig. 3C shows that *XPACE4* mRNA levels remain depleted throughout blastula and gastrula stages. By in situ hybridization, no RNA is detectable in oligo-depleted embryos at the late blastula stage, although a low level of nuclear transcript is visible by mid-gastrulation, indicating that zygotic transcription begins at this time (data not shown).

As mouse Nodal is a known substrate of mammalian PACE4 (Beck et al., 2002), we assessed the loss of activity of XPACE4 in a functional assay. For paracrine assays, control and *XPACE4*-depleted oocytes were injected with *Xnr1* mRNA and co-cultured with wild-type animal caps as described in the Materials and methods. Fig. 3D shows that the depletion of *XPACE4* decreases the inducing activity of Xnr1. Although the organizer genes, *chordin* (*Chd*) and *goosecoid* (*Gsc*), and the mesodermal gene *Xbra* and endodermal gene *XSox17α* are induced in animal caps co-cultured with control oocytes overexpressing *Xnr1* mRNA, caps cultured with *XPACE4*-depleted oocytes show little inducing activity. This indicates that the Xnr1-processing activity of endogenous XPACE4

Table 2. The gastrulation delay of *XPACE4*-depleted embryos

	Gastrulation	Normal (%)	Delayed (%)
Un		165 (95.9)	7 (4.1)
P(–)		8 (5.1)	149 (94.9)

Table 3. The phenotypes of *XPACE4*-depleted tailbuds

Tailbud phenotype	Normal (%)	Small head (%)	Headless (%)	Other (%)
Un	33 (78.6)	5 (11.9)	1 (2.4)	3 (7.1)
P(–)	4 (8.7)	22 (47.8)	12 (26.1)	8 (17.4)

protein is reduced by the antisense oligo-mediated depletion of the maternal *XPACE4* mRNA.

XPACE4 is required for mesoderm induction

To determine the function of maternal XPACE4 during embryogenesis, we used the host transfer technique to fertilize *XPACE4*-depleted oocytes. *XPACE4*-depleted embryos develop normally through the cleavage and blastula stages. The phenotype caused by maternal *XPACE4* depletion is obvious at gastrulation, when the formation of the blastopore is delayed compared with controls (Table 2). This phenotype is rescued by the reintroduction of *XPACE4* mRNA into sibling *XPACE4*-depleted embryos (Fig. 4A,B). After 1-2 hours delay, the blastopore ring forms and closes and the embryos continue to develop, with varying degrees of abnormality of anterior structures (Fig. 4C; Table 3). Similar phenotypes are observed when 30-45 ng of *XPACE4* morpholino oligo is used (data not shown).

As *XPACE4* depletion in oocytes abrogated the ability of injected *Xnr1* mRNA to induce mesoderm in animal caps (Fig.

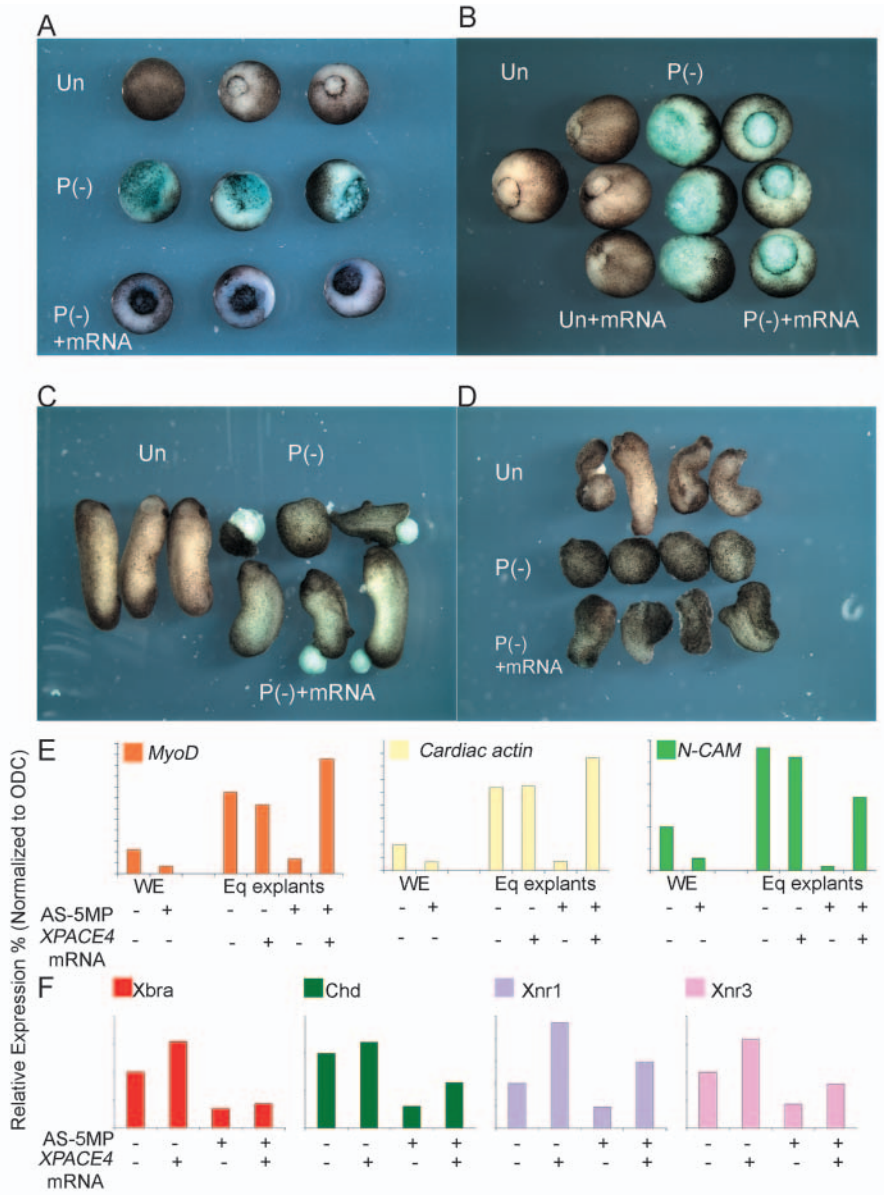


Fig. 4. Depletion of *XPACE4* disrupts gastrulation, mesoderm induction and normal development. (A) *XPACE4*-depleted embryos [P(–)] compared with controls (Un) show delay in gastrulation and rescue by *XPACE4* mRNA [P(–)+mRNA]. (B) Gastrulation delay of *XPACE4* depletion is rescued by the reintroduction of *XPACE4* mRNA. *XPACE4* mRNA injected into control embryos (Un+mRNA) speeds up gastrulation and rescues the gastrulation delay of *XPACE4*-depleted embryos [P(–)+mRNA]. (C) *XPACE4*-depleted embryos develop patterning defects and anterior abnormalities. Depletion of *XPACE4* [P(–)] results in a variety of late phenotypes ranging from ventralization to small heads. These phenotypes can be partially rescued by the reintroduction of *XPACE4* mRNA [P(–)+mRNA]. (D) Equatorial explants (Eq explants) of *XPACE4*-depleted embryos fail to elongate. Explants dissected from control embryos (Un) show normal convergent extension movements and elongation. *XPACE4*-depleted embryos [P(–)] show severe reduction in elongation; *XPACE4* mRNA rescues the elongation defect [P(–)+mRNA]. (E) Analysis of gene expression of equatorial explants from *XPACE4*-depleted embryos. Explants (Eq explants) dissected from control (AS-5MP–) and *XPACE4*-depleted (AS-5MP+) embryos at mid-blastula stage are cultured and analyzed together with stage 28 whole embryos (WE). The expression of general mesodermal marker *MyoD*, dorsal mesoderm marker *cardiac actin* and neural marker *NCAM* are all reduced in *XPACE4*-depleted whole embryos and explants. XPACE4 mRNA rescues the expression of markers. (F) Analysis of gene expression of *XPACE4*-depleted gastrulae. *XPACE4*-depleted whole embryos are harvested together with sibling control embryos. The expression of *Xbra, chordin* (*Chd*), *Xnr1* and *Xnr3* is analyzed using real-time RT-PCR. At stage 10.5, *XPACE4*-depleted embryos (AS-5MP+) show a reduction in the levels of these markers and the reduction is partially rescued by *XPACE4* mRNA. The mRNA alone increases marker levels. (For rescue experiments 75-100 pg of XPACE4 mRNA is injected vegetally into both cells at the two-cell stage.)

3D), we reasoned that *XPACE4*-depleted embryos may be deficient in Xnr signaling and therefore deficient in mesoderm induction. Four assays were used to test this.

First, equatorial regions, which are specified at the late blastula stage to form mesodermal tissue, were dissected from control and *XPACE4*-depleted embryos, cultured until the neurula stage and analyzed for the expression of mesodermal (*MyoD* and *cardiac actin*) and neural (*NCAM*) markers. Fig. 4D shows that control equatorial explants undergo the typical convergent extension movements indicative of mesoderm induction. By contrast, *XPACE4*-depleted equatorial explants are unable to undergo convergent extension movements, a deficiency that is specific to *XPACE4*-depletion as it is rescued by the reintroduction of *XPACE4* mRNA. The expression of both *cardiac actin* and *MyoD* expression is reduced by *XPACE4*-depletion and is rescued by the reintroduction of *XPACE4* mRNA (Fig. 4E). Neural tissue is normally secondarily induced in control equatorial explants. Neural induction assayed by the expression levels of *NCAM* is reduced by *XPACE4*-depletion and rescued by *XPACE4* mRNA. Marker analysis of gastrulae using real-time RT-PCR confirms that the expression of mes-endodermal genes is reduced in *XPACE4*-depleted embryos. In particular, the general mesodermal gene *Xbra*, and the organizer gene *chordin* (*Chd*) are reproducibly reduced in expression, while *Xnr1*, which is known to be positively autoregulated, and *Xnr3* are also affected. The expression of *Chd*, *Xnr1* and *Xnr3* is partially rescued by the reintroduction of *XPACE4* mRNA (Fig. 4F). The expression of the mesodermal markers *Fgf8* and *derrière* in whole embryos is little affected by *XPACE4*-depletion (data not shown).

Second, we asked whether endogenous mesoderm induction by vegetal masses was reduced in *XPACE4*-depleted embryos, using Nieuwkoop assays (Fig. 5A). Animal caps were dissected from wild type mid-blastula stage embryos and co-cultured for 1-2 hours with vegetal masses dissected from control or *XPACE4*-depleted embryos at the mid-blastula stage. After the induction period, the caps were separated and cultured until the mid-gastrula stage and analyzed for induced gene expression. Fig. 5A shows that the mes-endodermal genes *chordin* (*Chd*), *goosecoid* (*Gsc*), *Fgf8*, *Xbra* and *XSox17α*, which are targets of TGFβ signaling, are significantly induced in animal caps by wild-type vegetal masses, but not *XPACE4*-depleted vegetal masses. This experiment was repeated twice with similar results.

Third, we assayed the activity of ARE-luciferase reporter in *XPACE4*-depleted embryos. Control and *XPACE4*-depleted embryos were injected vegetally with the ARE-luciferase reporter at the two-cell stage and assayed for luciferase activity at stage 10.5. Fig. 5B shows average ARE-luciferase activity is significantly reduced in *XPACE4*-depleted embryos.

Fourth, we determined the phospho-Smad2 levels in *XPACE4*-depleted embryos by western blots using a phospho-Smad2 specific antibody. Fig. 5C shows that *XPACE4*-depleted embryos have decreased phospho-Smad2 levels most significantly at the late blastula.

These results indicate that XPACE4 regulates endogenous mesoderm induction.

XPACE4 regulates processing of specific TGFβ proteins during *Xenopus* embryogenesis

To determine which TGFβ family members expressed during early *Xenopus* embryogenesis are substrates for XPACE4, we assayed control and *XPACE4*-depleted embryos for processing of different TGFβ proteins. We injected mRNA coding for HA-tagged proteins into the vegetal cytoplasm of wild-type and *XPACE4*-depleted embryos at the two-cell stage, and assayed for precursor and mature forms of proteins by western blotting using a high-affinity HA antibody at the early gastrula stage. The HA tag was inserted into the C terminus of each construct, allowing the visualization of the mature protein, precursor protein and any stable C-terminal-containing intermediaries of Xnr2, Xnr1, Xnr3, Xnr5, Vg1, ActivinB and Derrière. In many cases, the analysis of whole embryo homogenates showed that only a small percentage of the overexpressed proteins were

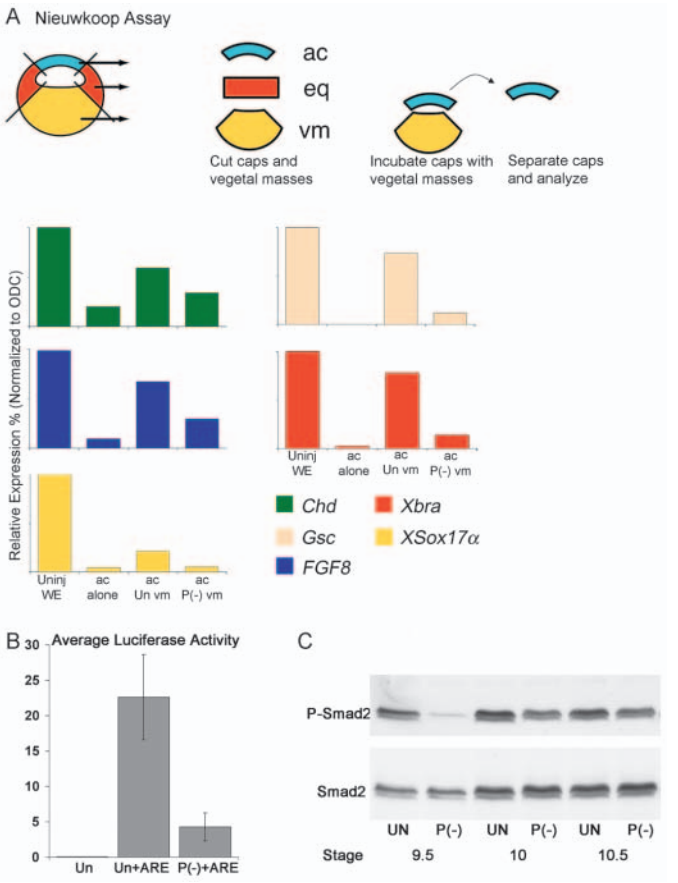


Fig. 5. Induction of mesoderm is disrupted by *XPACE4* depletion. (A) A schematic presentation of the Nieuwkoop Assay details the Materials and methods. The marker expression of animal cap explants (ac) co-cultured with control (Un vm) and *XPACE4*-depleted vegetal masses [P(–) vm] is analyzed by real-time RT-PCR. Animal caps incubated alone (ac alone) do not express significant levels of mes-endodermal genes. The induction of organizer genes *chordin*, *goosecoid*, mesodermal genes *Fgf8* and *Xbra*, and endodermal gene *XSox17α* are all reduced in caps cultured with *XPACE4*-depleted vegetal masses [ac P(–) vm] compared with controls (ac Un vm). Uninjected whole embryo (Uninj WE) is used for the dilution series and the quantification. (B) *XPACE4*-depleted embryos [P(–)+ARE] have reduced ARE-luciferase reporter activity compared with controls (Un+ARE). (C) Western blot analysis of control (UN) and *XPACE4*-depleted [P(–)] gastrulae show phospho-Smad2 levels are reduced by *XPACE4* depletion. Total Smad2 levels are shown as loading control.

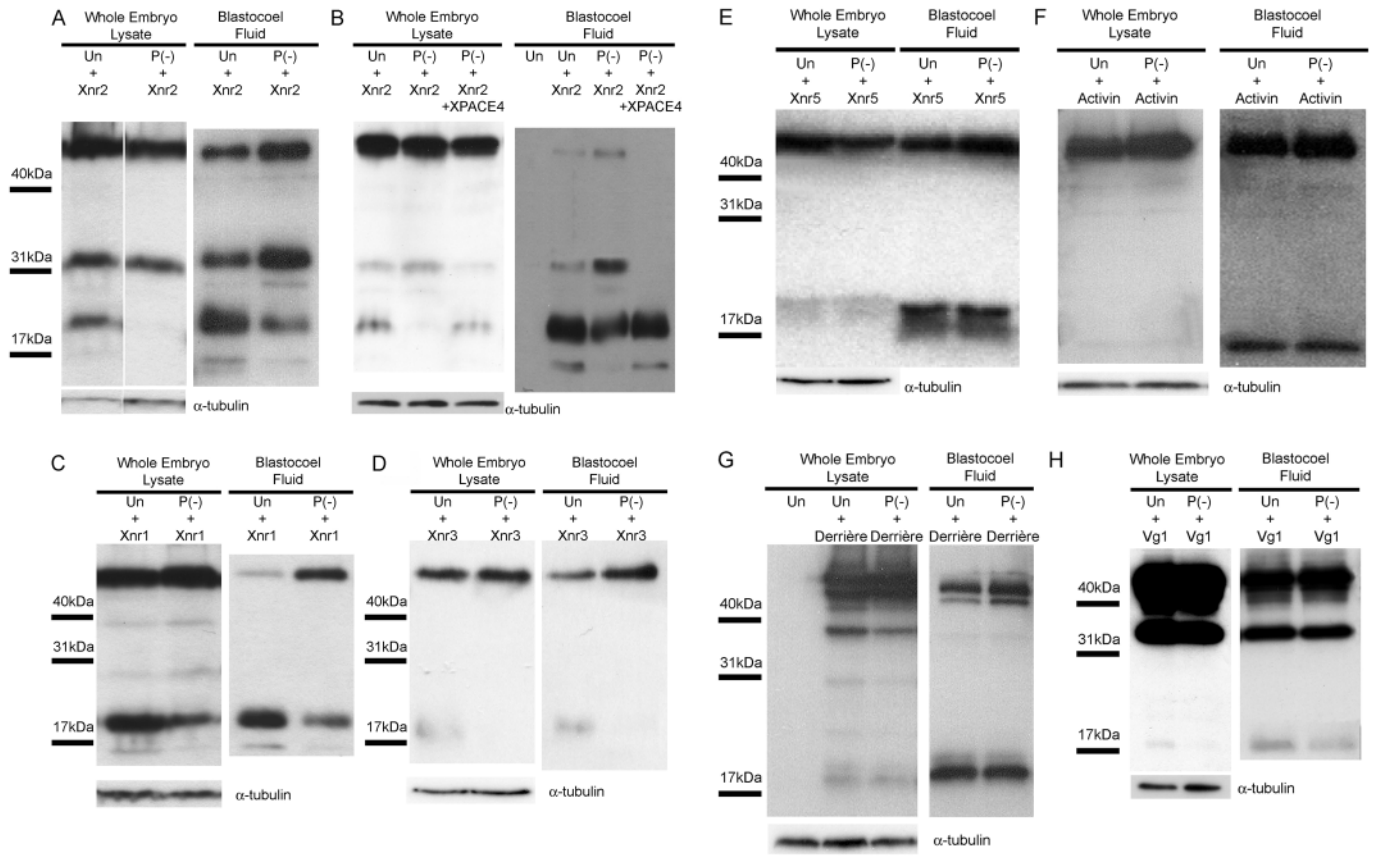


Fig. 6. XPACE4 depletion affects the processing of a specific subset of TGFβ proteins. (A) Maturation of Xnr2 is reduced in XPACE4-depleted embryos. Western blots from control (Un) and XPACE4-depleted [P(–)] whole embryos and blastocoel fluids overexpressing *Xnr2-HA* mRNA (200 pg) show the mature form of Xnr2-HA (26 kDa) is reduced in whole embryo homogenates of XPACE4-depleted embryos. In the blastocoel fluid of XPACE4-depleted embryos, a significant reduction in mature Xnr2 level and accumulation of the unprocessed (57 kDa) and an intermediate form (33 kDa) are detected. (B) Maturation defect of Xnr2 in XPACE4-depleted embryos is rescued by injection of *XPACE4* mRNA (150 pg). In whole embryos, the level of mature Xnr2 is rescued by *XPACE4* mRNA injection. In the blastocoel fluid the unprocessed and intermediate forms are rescued by *XPACE4* mRNA injection. (C) Maturation of Xnr1 is reduced in XPACE4-depleted embryos expressing 200 pg of *Xnr1-HA* mRNA. The mature form of Xnr1-HA (24 kDa) is reduced in whole embryo homogenates of XPACE4-depleted embryos. In the blastocoel fluid, a significant reduction in mature Xnr1 level and accumulation of the unprocessed form (57 kDa) are detected. (D) Maturation of Xnr3 is reduced in XPACE4-depleted embryos expressing 300 pg of *Xnr3-HA* mRNA. The mature form of Xnr3-HA (22 kDa) is reduced in whole embryo homogenates of XPACE4-depleted embryos. In the blastocoel fluid, a significant reduction in mature Xnr3 level is detected. (E) Maturation of Xnr5 is not affected in XPACE4-depleted embryo lysates or blastocoel fluids. *Xnr5-HA* mRNA (100 pg) is injected. (F) Maturation of ActivinB is not affected in XPACE4-depleted embryos. A very low level of mature ActivinB-HA (15 kDa) is more concentrated in the blastocoel fluid both in controls and XPACE4-depleted whole embryos. However, there is no significant change in levels of mature (15 kDa) or unprocessed forms (54 kDa) of ActivinB-HA. *ActivinB-HA* (400 pg) mRNA is injected. (G) Maturation of Derrière is not affected in XPACE4-depleted embryos. There is no significant change in levels of mature (21 kDa) or unprocessed (50 kDa) forms of Derrière-HA, either in whole embryo homogenates or in the blastocoel fluid of XPACE4-depleted embryos. *Derrière-HA* mRNA (400 pg) is injected. (H) Maturation of Vg1 is reduced in XPACE4-depleted embryos. The unprocessed Vg1 as a doublet (46 and 44 kDa), and intermediate form (35 kDa) are easily detected in whole embryo lysates and the blastocoel fluid. The mature form (18 kDa) is reduced in the blastocoel fluid of XPACE4-depleted embryos. *Vg1-HA* mRNA (600 pg) is injected. (α-tubulin is used as a loading control.)

cleaved, making comparison between control and *XPACE4*-depleted embryos difficult. Therefore, we took advantage of the fact that both precursor and cleaved forms of the HA-tagged TGFβ proteins are secreted into the blastocoel cavity (Williams et al., 2004). As this concentration of pro-protein and mature protein is uncontaminated with cellular debris, it provides a more sensitive assay for the determination of changes due to XPACE4-depletion. The experiments were repeated at least three times and a representative experiment is presented for each of TGFβ protein (Fig. 6A-H).

As Nodal processing is defective in mice lacking both furin and PACE4 (Beck et al., 2002), we first tested the effect of XPACE4-depletion on the cleavage of nodal related proteins Xnr1, Xnr2, Xnr3 and Xnr5. Fig. 6 shows the cleavage profile of overexpressed TGFβ proteins in embryo lysates and blastocoel fluid and Table 4 summarizes the effects of XPACE4 depletion on processing of different TGFβ proteins. In XPACE4-depleted whole embryo lysates, the mature Xnr2-HA is reduced compared with controls. Interestingly, the blastocoel fluid analysis shows the reduction in mature Xnr2-HA, and a relative increase in both the uncleaved and intermediate forms as a result of XPACE4 depletion. Thus, XPACE4 is required

Table 4. Summary for activity and processing of TGFβ proteins

TGFβ	mRNA dose required for dorsal mesoderm induction (*Gsc*, C*hd*)	mRNA dose required for general mesoderm induction (*Xbra*)	Cleavage by endogenous XPACE4	Proprotein [levels and M_r (kDa)]	Mature protein [levels and M_r (kDa)]	Secreted intermediaries [levels and M_r (kDa)]	References
Xnr5	>1 pg	0.3-1 pg	No	55	20	-	Takahashi et al., 2000
ActivinB	>2 pg	1-2 pg	No	54	15	-	Clements et al., 1999
Xnr2	>10 pg	1-10 pg	Yes	↑ 57	↓ 26	↑ 33	Clements et al., 1999; Jones et al., 1995; Eimon and Harland, 2002
Xnr1	>10 pg	10 pg	Yes	↑ 57	↓ 24	-	Clements et al., 1999; Jones et al., 1995
Derrière	>15 pg	15 pg	No	50	21	-	Sun et al., 1999
Xnr3	–	100-300 pg	Yes	↑ 53	↓ 22	-	Yokota et al., 2003
A-Vg1	>20 pg	20 pg	N/D	46	18	-	Kessler and Melton, 1995
B-Vg1	>20 pg	20 pg	N/D	46	18	-	Clements et al., 1999; Kessler and Melton, 1995; Thomsen and Melton, 1993
Vg1(Ser20)	>500 pg	500 pg	Yes	↑ 46/44	↓ 18	35	
Vg1(Pro20)	–	–	N/D	45/43.5	17	-	Dale et al., 1989; Tannahill and Melton, 1989

N/D, not determined; ↑, accumulates in *XPACE4*-depleted embryos; ↓, decreases in *XPACE4*-depleted embryos.

for the production of mature Xnr2-HA but not for the formation of the intermediate form. The reduction in cleavage of Xnr2-HA is specifically due to XPACE4 depletion, as Fig. 6B shows that the amount of mature protein increases and the uncleaved and the intermediate forms decrease when *XPACE4* mRNA is injected at the two- to four-cell stage, along with *Xnr2-HA* mRNA into XPACE4-depleted embryos. Processing of Xnr1 (Fig. 6C) and Xnr3 (Fig. 6D) is also reduced by XPACE4 depletion. In contrast to Xnr1, Xnr2 and Xnr3, cleavage of Xnr5-HA (Fig. 6E), ActivinB (Fig. 6F) or Derrière (Fig. 6G) is not affected by XPACE4 depletion.

In contrast to the zygotically expressed Xnr proteins and derrière, Vg1 is a maternal mRNA and protein, which is, like XPACE4, localized to the vegetal cortex of *Xenopus* oocytes (Dale et al., 1989; Melton, 1987; Tannahill and Melton, 1989; Weeks and Melton, 1987). Next, we asked whether Vg1 processing would be affected by *XPACE4* depletion. HA-tagged *Xenopus* Vg1 was cloned from oocyte cDNA as described in the Materials and methods, and found to have a serine instead of a proline residue in position 20 (AY838794) compared with the published sequence (Weeks and Melton, 1987). Detailed analysis of the ESTs available for *Xenopus laevis* revealed that this serine residue is conserved in 14 ESTs compared with 13 ESTs with proline residues, suggesting that this represents a pseudoallelic form of Vg1. Western blot analysis of overexpressed Vg1-HA in *Xenopus* embryos shows the processing of Vg1-HA is affected in *XPACE4*-depleted embryos, as the mature protein levels decrease and there is a corresponding increase in the levels of unprocessed forms (Fig. 6H). The intermediate form of Vg1 may be a nonspecific degradation product or product of cleavage at an upstream site. Native Vg1 has been shown to have no mesoderm inducing activity. As our sequence is different from the published one, we tested the mesoderm inducing activity of Vg1 and found that this Vg1 has weak general mesoderm inducing activity (data not shown).

These results demonstrate that endogenous XPACE4 cleaves exogenous Xnr1, Xnr2, Xnr3 and Vg1, but not ActivinB, Derrière and Xnr5. Taken together, this work provides the first evidence that XPACE4 is an essential vegetally localized regulator of mesoderm induction in *Xenopus* embryos, with specificity towards the TGFβs Xnr1, Xnr2, Xnr3 and Vg1.

Discussion

Several studies have shown that PCs when overexpressed have the ability to cleave a wide spectrum of substrates. However, the specificity of PACE4 towards endogenous substrates during development has not been investigated. We have studied this, using loss-of-function assays, in early *Xenopus* embryos. Three novel observations are made. First, maternal *XPACE4* mRNA is localized to the mitochondrial clouds and vegetal hemispheres of oocytes. Second, it is required for the endogenous mesoderm inducing activity of vegetal cells before gastrulation, and third, it has substrate-specific activity, cleaving Xnr1, Xnr2, Xnr3 and Vg1, but not Xnr5, Derrière and ActivinB pro-proteins.

XPACE4 and its mammalian ortholog are localized mRNAs

At the late blastula and early gastrula stages, the expression domains of *XPACE4* and several TGFβ mRNAs, including Vg1, Xnr1 and Xnr2, coincide. In mouse, processing depends on the ability of the secreted pro-protein precursor and the

PACE4/SPC4 enzyme to contact each other in the extracellular space (Beck et al., 2002; Constam and Robertson, 1999). We have no direct evidence that endogenous XPACE4 is a secreted protein, as specific antibodies are not available. However, we find that uncleaved as well as cleaved forms of exogenous Vg1, Xnrs, ActivinB and Derrière accumulate in a stable fashion in blastocoel fluid, and that, for Xnr1, Xnr2 and Xnr3, the uncleaved pro-proteins accumulate more when XPACE4 is depleted. The simplest explanation for this is that XPACE4 is secreted. Thus, the pro-proteins are normally cleaved extracellularly by secreted XPACE4, and accumulate more in the blastocoel in XPACE-depleted embryos, because the enzyme is not there to process it. However, this remains to be confirmed directly. Another possibility is that cleavage of endogenous TGFβs occurs intracellularly, but that excess uncleaved and intermediate forms are produced and secreted in blastocoel fluid as a result of the excessive loading of the cells with these mRNAs (100-600 pg of mRNA for HA-tagged TGFβs was injected). It will be important to determine whether endogenous XPACE4 is present in blastocoel fluid when antibodies become available.

Later in development, *XPACE4* mRNA is found in the notochord, the brain and the olfactory bulb, where its position correlates with expression of rodent PACE4 in the developing CNS (Akamatsu et al., 1997; Zheng et al., 1997), and in a subset of cells in the endoderm correlating with the position of primordial germ cells. Possible roles of XPACE4 in these locations remain to be determined.

XPACE4 is required for mesoderm induction

The regulation of TGFβ signaling in vertebrate embryogenesis is crucial for correct tissue formation, and this study together with work in mouse embryos shows the importance of subtilisin-family proteases in this process (Beck et al., 2002; Constam and Robertson, 1999; Constam and Robertson, 2000). Although the targeted loss of function of PACE4 in mouse embryos showed relatively mild effects [causing only 25% of animals to die prenatally with cardiac malformations, laterality defects and craniofacial abnormalities, and 75% to survive to birth (Constam and Robertson, 2000)], PACE4/furin double mutants showed severe anterior visceral endoderm and axis formation defects, indicative of disruption of Nodal cleavage and function (Beck et al., 2002). In the studies presented here, we find that the predominant contribution of *XPACE4* mRNA is from maternal stores accumulated during oogenesis, with relatively small and localized areas of synthesis of zygotic XPACE4 transcript. Two observations argue that the maternal store of protein accumulated specifically in vegetal, endo-mesoderm precursors is stable and long lived. First, the XPACE4 phenotype, delayed gastrulation and headless development, was more pronounced the longer the oligo-injected oocytes were incubated before fertilization (48 hours versus 24 hours). Longer incubation after oligo injection would be expected to produce greater turnover of the stable XPACE4 protein pool synthesized during oogenesis. Second, we found that a morpholino oligo against XPACE4 was effective in causing the same phenotype as AS-5MP oligo if the morpholino oligo was injected 2 days before fertilization, but was not effective when injected into fertilized eggs (data not shown). It will be interesting to determine if there is also a maternal contribution of PACE4/SPC4 protein in mouse development, which allows the relatively normal early development of mPACE4$^{-/-}$ embryos.

Several lines of evidence presented show the importance of XPACE4 in mesoderm induction. First, loss of XPACE4 causes a loss of mesodermal markers in whole embryos. Second, Niewkoop assays show that XPACE-depleted vegetal masses are unable to induce wild-type animal caps to form mesoderm. Third, loss of XPACE4 causes loss of ARE-luciferase activity concomitant with loss of mesodermal gene expression. Last, phospho-Smad2, the immediate downstream target of activin-type TGFβ signaling, is also reduced in XPACE4-depleted embryos. All approaches show the reduction of Nodal signaling activity due to the depletion of maternal XPACE4. Since, of the TGFβs tested, we found that Xnr1, Xnr2, Xnr3 and Vg1 are substrates for XPACE4 cleavage, it is likely that the defect in mesodermal induction due to XPACE4 depletion is caused by the reduction in mature forms of these proteins. Previously, we have shown that Xnr3 is required for convergence extension movements and head formation (Yokota et al., 2003), and that Xnr1 and Xnr2 are able to rescue axis formation and mesoderm induction in embryos depleted of TGFβ signaling by antisense ablation of maternal VegT (Kofron et al., 1999; Xanthos et al., 2001; Zhang et al., 1998). We find here that the Vg1-like protein Derrière is not a substrate for XPACE4. Derrière is known to be important in establishment of tail and posterior axial structures, and rescues VegT-depleted embryos to the extent of causing late gastrulation and the formation of tails and dorsal axes but not heads (Kofron et al., 1999). Thus, one explanation for the late gastrulation and posterior axial development of XPACE4-depleted embryos is that normal Derrière function is maintained. This hypothesis is supported by the fact that phospho-Smad2 levels approach control levels by mid-gastrulation when Derrière is expressed (Fig. 5C). It will be interesting to determine whether Derrière, ActivinB and Xnr5 are substrates for the other maternally supplied PCs.

Comparisons of cleavage spectra and activities of HA-tagged TGFβ proteins

To date, this work provides the most comprehensive comparison of the cleavage spectra of seven TGFβ proteins in embryo lysates and blastocoel fluids, and the effect of depletion of one of the regulating enzymes on those patterns. As the HA tags were placed at the C termini of the proteins, N terminal pro-domain fragments were not visualized. Table 4 correlates these findings with the known biological activity of each of the proteins as measured by mesoderm induction. Several conclusions can be drawn from the comparisons.

First, XPACE4 regulates Xnr1, Xnr2, Xnr 3 and Vg1 but not Xnr5, Derrière and ActivinB. As all of these proteins (with the exception of ActivinB) share an RXXR consensus cleavage motif, the substrate specificity of XPACE4 may be due to different residues flanking this consensus site. Specificity and optimum activity of convertases have been documented to be dependent on these flanking sequences (Apletalina et al., 1998; Henrich et al., 2003), and studies on Vg1 cleavage indicate that the presentation of the cleavage site in a particular context and/or conformation is crucial for regulating cleavage (Dohrmann et al., 1996; Thomsen and Melton, 1993).

Second, we show here that the mature form of *Xenopus* Vg1 can be detected by western blots using a high-affinity HA

antibody, that this form has weak mesoderm-inducing activity and that the processing of Vg1 is reduced in *XPACE4*-depleted embryos. Previously, although native Vg1 protein has been shown to be present vegetally in oocytes and early *Xenopus* embryos (Dale et al., 1989), the mature form was neither detected nor active in mesoderm induction (Tannahill and Melton, 1989). In addition, while Vg1 chimeras with prodomains of either Activin or BMP proteins were processed efficiently and were potent mesoderm inducers (Dale et al., 1993; Thomsen and Melton, 1993), mature exogenous Vg1 could be detected but had no mesoderm-inducing activity (Kramer and Yost, 2002). By contrast, the zebrafish homolog of Vg1, zDVR-1 (Dohrmann et al., 1996) and chick Vg1 (Shah et al., 1997) were detectable as mature proteins with weak mesoderm-inducing activity in *Xenopus* oocytes.

The Vg1 used here has some sequence differences compared with the published Vg1 sequence. One difference is a C-to-T transition resulting in a serine residue (TCN codon) instead of a proline (CCN codon) at position 20. The analysis of available EST sequences has suggested that both of these alleles are present in *Xenopus* oocytes. The proline residue in the helix rich N terminal region of Vg1 may be responsible for disruption of the structure, and affect processing and render the protein functionless. This may explain the previous observation that N-terminal signal peptide of Vg1 does not get cleaved and Vg1 protein fails to dimerize (Dale et al., 1993), whereas the Vg1 with a serine residue at position 20 has weak mesoderm-inducing activity (data not shown). It will be interesting to determine the function of this Vg1 allele in detail and to understand the structural distinctions between the different forms.

Third, the Xnr2 cleavage spectrum is more complex than that of the other six proteins. Although the intermediate form of 33 kDa may be a degradation product or caused by nonspecific cleavage at an upstream cryptic site, the fact that it accumulates in an XPACE-depleted background suggests that it may be formed by the specific pro-protein digestion activity of another endoprotease, generating a processing intermediate that is subsequently cleaved at the known downstream site. We have shown previously that BMP4 processing involves sequential cleavage that is important in regulating the stability and activity of the mature protein (Cui et al., 2001). Analysis of Xnr2 protein sequence reveals the presence of a consensus RXXR motif located ~70 amino acids upstream of the known cleavage site that would be predicted to yield a protein form of ~33 kDa upon endoproteolysis. Xnr2 generated from a precursor in which this upstream site is mutated, however, maintains reduced activity, suggesting that processing at the downstream site occurs independent of the upstream site and is sufficient for Xbra induction activity. A double cleavage mutant of Xnr2 that fails to generate any mature protein also induces Xbra, suggesting that unprocessed Xnr2 has some signaling activity (Eimon and Harland, 2002).

Fourth, as has been shown previously for Activin and Vg1, there is no simple correlation between the amount of mature form of each of the TGFβs and their biological activity. Here, Xnr5 and Activin are the most active proteins. They work at very low doses of RNA in mesoderm induction assays and yet accumulate less well than Xnr1 and 2 in whole embryo lysates. They do, however, accumulate substantially in blastocoel fluid. Cleaved forms of Xnr3 and Vg1 are only very weakly detected in either the lysates or the blastocoel fluid. Undoubtedly, the regulation of each TGFβ is complex and individual, involving the interplay of stabilizing or competing intermediary cleavage forms, other competing co-expressed TGFβ family members and the presence or absence of localized co-receptors. Specific loss-of-function and mutation analysis for each individual TGFβ and detailed functional analysis of the other endoproteolytic enzymes are required to fully understand their function.

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