Development 126, 1467-1482 (1999)
Printed in Great Britain © The Company of Biologists Limited 1999
DEV3974

derrière: a TGF-β family member required for posterior development in *Xenopus*

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Accepted 25 January; published on WWW 3 March 1999

SUMMARY

TGF-β signaling plays a key role in induction of the *Xenopus* mesoderm and endoderm. Using a yeast-based selection scheme, we isolated *derrière*, a novel TGF-β family member that is closely related to *Vg1* and that is required for normal mesodermal patterning, particularly in posterior regions of the embryo. Unlike *Vg1*, *derrière* is expressed zygotically, with RNA localized to the future endoderm and mesoderm by late blastula, and to the posterior mesoderm by mid-gastrula. The *derrière* expression pattern appears to be identical to the zygotic expression domain of *VegT* (*Xombi, Brat, Antipodean*), and can be activated by *VegT* as well as *fibroblast growth factor* (*FGF*). In turn, *derrière* activates expression of itself, *VegT* and *eFGF*, suggesting that a regulatory loop exists between these genes. *derrière* is a potent mesoderm and endoderm inducer, acting in a dose-dependent fashion. When misexpressed ventrally, *derrière* induces a secondary axis lacking a head, an effect that is due to dorsalization of the ventral marginal zone. When misexpressed dorsally, *derrière* suppresses head formation. *derrière* can also posteriorize neurectoderm, but appears to do so indirectly. Together, these data suggest that *derrière* expression is compatible only with posterior fates. In order to assess the in vivo function of *derrière*, we constructed a dominant interfering Derrière protein (Cm-Derrière), which preferentially blocks Derrière activity relative to that of other TGFβ family members. Cm-*derrière* expression in embryos leads to posterior truncation, including defects in blastopore lip formation, gastrulation and neural tube closure. Normal expression of anterior and hindbrain markers is observed; however, paraxial mesodermal gene expression is ablated. This phenotype can be rescued by wild-type *derrière* and by *VegT*. Our findings indicate that *derrière* plays a crucial role in mesodermal patterning and development of posterior regions in *Xenopus*.

Key words: *Xenopus*, TGF-β, *derrière*, *Vg1*, *VegT*, Mesoderm, Posterior, Dominant negative

INTRODUCTION

Members of the TGFβ gene family are key regulators of mesoderm determination in *Xenopus*, as suggested by their ability to induce mesoderm in explanted tissue (Harland and Gerhart, 1997) and by the ablation of mesoderm after expression of dominant negative TGF-β receptors in whole embryos (Hemmati-Brivanlou and Melton, 1992). Maternally expressed TGF-βs include *Vg1* whose RNA is localized to the vegetal hemisphere and is therefore in the correct position to induce mesoderm (Weeks and Melton, 1987).

In conjunction with maternally expressed *β-catenin* (Heasman, 1997), TGF-β signaling may also direct dorsoventral (D/V) mesodermal patterning, as suggested by the ability of different concentrations of Activin to induce a graded readout of D/V mesodermal fates in *Xenopus* explants (Green et al., 1992). Zygotically expressed TGF-β family members that may be involved in D/V mesodermal patterning include the *Xnr* genes (*Xnr1*, *Xnr2*, *Xnr3* and *Xnr4*), whose RNAs are either localized to the entire marginal zone or restricted dorsally to the organizer (Ecochard et al., 1995; Jones et al., 1995; Smith et al., 1995; Lustig et al., 1996a; Joseph and Melton, 1997). Additionally, Bone Morphogenetic Proteins (BMPs) are TGF-β family members that may act both maternally and zygotically to promote ventral fates (Harland, 1994).

Closely linked to D/V mesodermal patterning is formation of the anteroposterior (A/P) axis, which appears in the mesoderm by early gastrula (Zoltewicz and Gerhart, 1997) and, soon after, in the ectoderm (Saha and Grainger, 1992; Sive et al., 1989; Gamse and H. L. S., unpublished data). Inhibition of BMP signaling has been implicated in anteroposterior axis determination, since ventral expression of a dominant negative BMP receptor (Graff et al., 1994; Suzuki et al., 1994: Glinka et al, 1997) or a dominant negative BMP4 ligand (Hawley et al., 1995) leads to formation of a secondary axis that never contains a head. Additionally, non-BMP TGF-βs including *Xnrs*, *activin* and an activated form of *Vg1* are also able to

induce, in normal embryos, a secondary axis lacking a head (Thomsen et al., 1990; Dale et al., 1993; Jones et al., 1995; Lustig et al., 1996a; Joseph and Melton, 1997). Thus, in the context of the normal embryo, posterior fates can be activated either by antagonizing BMP signaling or by promoting non-BMP TGF-β signaling. Despite these intriguing data, however, a normal role for TGF-β family members in posterior patterning has not been defined, since no TGF-β with localized expression or endogenous activity consistent with such a role has been described.

We have begun to address the role TGF-β molecules normally play in A/P axis determination. In a yeast-based selection for signal sequence-containing proteins (Jacobs et al., 1997) expressed during *Xenopus* A/P patterning, we isolated a novel member of the TGF-β superfamily, Derrière. Mature Derrière protein is very similar to Vg1, however, unlike *Vg1*, *derrière* is both zygotically expressed and, by mid-gastrula, its expression is restricted to posterior mesoderm. The *derrière* expression pattern is identical to the zygotic expression of the T box gene, *VegT* (*Xombi*, *Brat*, *Antipodean*, hereafter called *VegT*, Stennard et al., 1996; Zhang and King, 1996; Lustig et al., 1996b; Horb and Thomsen, 1997), and appears to be activated in a regulatory loop with this gene. We demonstrate that *derrière* activity is required for normal mesodermal patterning and posterior development of the *Xenopus* embryo. Our findings indicate that TGF-β family members not only play important roles in D/V axis determination, but that one member of this family, *derrière*, plays a crucial role in posterior axial patterning.

MATERIALS AND METHODS

Embryos and explants

Methods for obtaining embryos are described in Sive et al. (1989). Microdissection was performed as described (Kolm et al., 1997). The starting materials for construction of a random-primed cDNA library that went through the yeast selection (see below) consisted of dorsal mesoderm and dorsal ectoderm of stage 11.5 embryos. The anterior limit was the anterior end of the archenteron and the posterior limit was the blastopore lip. The width of the explants was about 120° centered on the dorsal midline. For animal caps, animal hemisphere ectoderm was isolated within the window of 45 minutes centered around stage 8.5 and subsequently incubated as indicated. Anterior dorsal ectoderm (aDE) was isolated from stage 11 or 11.5 embryos. The anterior limit was the edge of the blastocoel, and the posterior limit was the mid-point between the anterior limit and the blastopore lip. The width of the explants was about 50° to each side of the dorsal midline.

Construction of libraries

For the random-primed cDNA library for the yeast selection, total RNA (600 μg) was isolated from the dorsal mesoderm/ectoderm explants using Proteinase K method followed by DNase I treatment (Gammill and Sive, 1997), poly(A)+ RNA was isolated using an oligo(dT) (Collaborative Biochemical Products) column. 0.5 μg poly(A)+ RNA was used for first- and second-strand cDNA synthesis (GIBCOBRL SuperScript Choice System). A random nanomer with an internal *Xho*I site (5′-AAGCTTGGCGGTCTCGAGNNNN-NNNNN-3′) was used at 60 μM for random priming. After second-strand synthesis, double-stranded cDNA was size-selected (300-600 bp) by PAGE and electroeluted before adapter ligation. Two oligos were used to form an *Eco*RI adapter: BIS1, 5′-AATTCGG-ACTACTACAGGTG-3′, unphosphorylated and BIS2, 5′-CACCT-GTAGTAGTCCG-3′, phosphorylated. cDNA was then separated from free adapters by PAGE, electroeluted and was subjected to PCR using phosphorylated BIS1. T4 DNA polymerase (in the absence of dATP and dTTP) was used to generate a cohesive *Eco*RI end at the 5′ end of cDNA before *Xho*I digestion at the 3′ end. The cDNA was directionally cloned into the SST vector (pSUC2T7M13ORI, Jacobs et al., 1997) for the yeast selection. For the full-length cDNA library, 300 whole embryos between stages 11.5 to 12 were harvested for total RNA. Poly(A)+ RNA was subsequently isolated and double-stranded cDNA synthesized using the same methods described above except the following: (1) an oligo(dT) primer with a *Xho*I site, 5′-CTCGAGTTTTTTTTTTTT-3′, was used along with methylated dCTP for first-strand synthesis; (2) two oligos were used for *Eco*RI adapter: 5′-AATTCCCATAGCAACAAACAGTA-3′ and 5′-TACT-GTTTGTTGCTATGGG-3′; and (3) no PCR amplification was performed and size selection (0.5 kb and above) and electroelution were not done until after adapter ligation and *Xho*I digestion. This cDNA was cloned directionally into *Eco*RI-*Xho*I double-digested CS2+ vector. The resulting library contains 5×10^6 independent clones and has an average insert size of 1.3 kb.

Yeast selection

The random-primed cDNA library was subjected to a yeast selection as described previously (Jacobs et al., 1997). In brief, the plasmid library was transformed into a yeast strain deleted for its endogenous invertase gene. The plasmid vector used to construct the library carried a modified invertase gene lacking its signal sequence. When a heterologous cDNA encoding a signal sequence was fused appropriately upstream of this defective invertase, the yeast's ability to grow on restrictive media was restored. The original *derrière* partial cDNA clone contained 282 base pairs. cDNA sequence was determined and used for database search. The full-length cDNA clone was isolated by standard hybridization with probe synthesized using oligo 5′-GAAAGTGATAGCCACAACTCTGCCATG-3′. The GenBank accession number for *derrière* is AF065135.

Isolation of RNA and northern analysis

Total RNA was prepared by Proteinase K method and analyzed by northern analysis as described (Kolm and Sive, 1995). Antisense derrière probe was prepared by asymmetric PCR amplification (Sive and Cheng, 1991) of derrière/CS2+ plasmid linearized with *Eco*RI using the primer 5′-CCCTTAGCATTTCCGTCAGT-3′.

In vitro transcription of capped RNAs and in situ hybridization probes

Capped RNAs for microinjection were transcribed in vitro as described (Kolm and Sive, 1995). *β-globin* and *lacZ* were as described (Kolm et al., 1997). Other templates were as follows: *Eco*RI linearized pSP64TBVg1 (for *BVg1*; Thomsen and Melton, 1993), pβB-64T (for *activin*; Sokol et al., 1991), pXFD/Xss (for XFD, Amaya et al., 1991); *Not*I linearized Xnr1/CS2+ (for *Xnr1*; Lustig et al., 1996a), pCS2+VegTfc (for *VegT*, Zhang and King, 1996), pdor3 (for *Xnr3*, Smith et al., 1995), derrière/CS2+ and Cm-derrière/CS2+; *Sma*I linearized Xbra/pSP64T (for *Xbra*, Smith et al., 1991); *Xba*I linearized Xnr4-64TNE (for *Xnr4*, Joseph and Melton, 1997); *Kpn*I linearized pCS2+Xnr2 (for *Xnr2*; Jones et al., 1995) followed by Klenow treatment. All were transcribed with SP6 RNA polymerase.

In situ hybridization probes were labeled with digoxygenin-11-UTP (Harland, 1991). *Krox20*, *en-2*, *otx2*, *HoxB9* and *XCG* were as described (Kolm et al., 1997). Other templates were as follows: derrière/CS2+, *Eco*RI linearized, T7 transcribed; pBSAC100 (for *m-actin*), *Eco*RI linearized, T3 transcribed; pSP70-N1 (for *NCAM*; (Kintner and Melton, 1987), *Eco*RV linearized, SP6 transcribed.

Microinjection

Microinjections were done as described (Kolm et al., 1997). See Figure Legends for stages and sites of injection. 80 pg *lacZ*-capped

RNA was included where necessary. Within each set of experiments, *β-globin*-capped RNA was used to make up for differences in amount of test RNA injected such that all embryos received the same total amount of RNA.

β-galactosidase staining and in situ hybridization

β-galactosidase staining was performed as described (Kolm and Sive, 1995). Whole-mount in situ hybridization was performed as described (Harland, 1991), with modifications described in Bradley et al. (1996). For in situ hybridization done on sectioned embryos, whole embryos were fixed for 1 hour first before being sectioned with eyebrow knife and fixed for another hour.

Relative quantitative RT-PCR

RNA and cDNA samples were prepared as described (Kolm et al., 1997). The optimal cycle numbers (listed below) were determined by titration using whole-embryo cDNA from similar stages. The PCR program used was: 95°C for 30 seconds, 55°C for 40 seconds and 72°C for 40 seconds. *NCAM* (28 cycles), *en-2* (26 cycles) and *Krox20* (26 cycles) primers were as described (Hemmati-Brivanlou and Melton, 1994). *HoxA7* (26 cycles), *m-actin* (22 cycles), *HoxB9* (27 cycles) and *Xcad3* (25 cycles) primers were as described (Kolm and Sive, 1997). *XCG* (17 cycles) primers were as described (Gammill and Sive, 1997). The sequences of other primers used are as follows (sense primer first): *ODC* (21 cycles): 5′-CAACGTGTGATGGGCTGGAT-3′ and 5′-CATAATAAAGGGTTGGTCTCTGA-3′, *Xbra* (24 cycles): 5′-TTCTGAAGGTGAGCATGTCG-3′ and 5′-GTTTGACTTTGC-TAAAAGAGACAGG-3′, *VegT* (28 cycles): 5′-TTAGCTTCCCAG-AGACAGAGT-3′ and 5′-CACATATAGCCTTGGGGAAATC-3′, *eFGF* (27 cycles): 5′-CGGGTTTCATATCCAGGTTTTAC-3′ and 5′-GCGTTATAGTTGTTGGGCAGAAG-3′, *gsc* (26 cycles): 5′-GGATTTTATAACCGGACTGTGG-3′ and 5′-TGTAAGGGAGCA-TCTGGTGAG-3′, *eomes* (25 cycles): 5′-GGGCCAACAGCAC-AAGAAATAC-3′ and 5′-TGGAGGCGCATAAGGGAAGAT-3′, *Xlim-1* (25 cycles): 5′-GTGTCTGCCTTCTATTCTCCTAA-3′ and 5′-GCACAGCCCGCACACTTGGTA-3′, *Pintallavis* (25 cycles): 5′-GCAGGCACCCAACAAGATGAT-3′ and 5′-CCAGATTCGGG-GTGCAGAGT-3′, *Xnot* (26 cycles): 5′-CAGACCTGCCTCCAAA-CTATCC-3′ and 5′-TCTCCCCTGGGCATCCTCATT-3′, *siamois* (27 cycles): 5′-AGGAACCCCACCAGGATAAAT-3′ and 5′-GTTG-ACTGCAGACTGTTGACTA-3′, *Xvent-1* (25 cycles): 5′-GCATC-TCCTTGGCATATTTGG-3′ and 5′-TTCCCTTCAGCATGGTTCAA-C-3′, *XK81* (19 cycles): 5′-TCATTCCGTTCCAGCTCTTCTTAC-3′ and 5′-TCCAGGGCTCTTACTTTCTCCAG-3′, *Xsox17α* (25 cycles): 5′-CAATGGCAGCTACCCTCACC-3′ and 5′-CTTGGCCACATAG-CTCAGATAC-3′, *endodermin* (21 cycles): 5′-TATTCTGACT-CCTGAAGGTG-3′ and 5′-GAGAACTGCCCATGTGCCTC-3′, *derrière* (22 cycles): 5′-TGGCAGAGTTGTGGCTATCA-3′ and 5′-CTATGGCTGCTATGGTTCCTT-3′.

Recombinant Derrière production

Three sets of oligonucleotide duplexes were obtained that encoded the mature region of Derrière (residues 241-354) from after the presumed maturation cleavage site to the stop codon and included *Nde*I and *Xba*I restriction sites at the 5′/3′ ends of *derrière*. This allowed cloning of the ligated duplexes into *Nde*I-*Xba*I-restricted *E. coli* expression vector pAL981 (LaVallie et al., 1993) with an in-frame fusion of *derrière* to an ATG. The codons of *derrière* were modified to reflect codon usage found in highly expressed *E. coli* genes. The *derrière* expression plasmid was sequenced and used to transform the *E. coli* strain GI934 (Lu et al., 1996). A fresh overnight culture of GI934 containing the *derrière* expression plasmid was used to inoculate IMC/Amp medium (M9 media containing 0.2% casamino acids, 0.5% glucose, 1 mM $MgSO_4$ and 100 μg/ml ampicillin) to an OD_{550} of 0.05. The culture was grown at 30°C until the OD_{550} reached 0.5, then L-tryptophan was added to a concentration of 100 μg/ml and the culture temperature shifted to 37°C. 4 hours later the cells were harvested and stored at −80°C until use. Recombinant Derrière expressed in *E. coli* was refolded to produce active dimers essentially as described (Schlunegger et al., 1992). In brief, the monomeric denatured protein was isolated from inclusion bodies by acidification and purified by size exclusion chromatography. Derrière protein was refolded at pH 8.5 using 2% CHAPS, 1.0 M NaCl and a glutathione redox couple. Final purification was carried out on a Mono S column (Pharmacia Biotechnology Inc.) and reversed-phase HPLC.

Factor treatments

For protein treatments, explants (both animal caps and aDE) were incubated at 20°C until desired stages. BSA (New England Biolabs) was used at 300 ng/ml in 0.5× MBS; bFGF (Promega) was used at 100 ng/ml (plus 200 ng/ml BSA) in 0.5× MBS; Derrière was used at 200 ng/ml (plus 100 ng/ml BSA) in 0.5× MBS. A co-treatment of bFGF and Derrière consisted of 100 ng/ml bFGF and 200 ng/ml Derrière.

Construction of Cm-*derrière*

The strategy for generating *derrière* cleavage mutant (Cm-*derrière*) is as described previously (Hawley et al., 1995). The two primers used to bring in substitutions were: 5′-GCATGGTCGACGGGAGTACT-CATTCATCACCTC-3′ (sense, paired with T7 primer) and 5′-GC-ATGGTCGACGCCTTGAGTTTTGCAATTGGATG-3′ (antisense, paired with SP6 primer) using derrière/CS2+ as template. The resulting 5′ (*Eco*RI-*Sal*I double-digested) and 3′ (*Sal*I-*Xho*I double-digested) ends of Cm-*derrière* were subcloned into CS2+ (*Eco*RI-*Xho*I double-digested) by a three-piece ligation and the final construct was confirmed by sequence determination.

RESULTS

derrière is a new member of the TGF-β superfamily

We isolated *derrière* using a yeast-based selection assay for secreted proteins, from a cDNA library prepared from *Xenopus* mid-gastrula dorsal mesoderm and ectoderm (Jacobs et al., 1997; see Materials and Methods). Based on sequence identity, potentially interesting clones were screened for localized expression by in situ hybridization at late gastrula. *derrière* showed localized posterior expression in this assay.

The *derrière* cDNA encoded a protein of 354 amino acids (Fig. 1A) and represented a previously unknown member of the TGF-β superfamily. A putative signal sequence cleavage site was present at the amino terminus (von Heijne, 1986), a maturation processing sequence (RAKR, Rehemtulla and Kaufman, 1992) was present at position 237 and, in the carboxy terminus, seven cysteines were present that are conserved among other TGF-β molecules (Kingsley, 1994). *derrière* is a member of the *Vg*-related family of TGF-βs and is most similar to *Xenopus Vg1* (56% identity for the full-length protein and 79% within the mature region; Fig. 1B). The percentage identities between the mature region of Derrière and of other Vg1-related proteins shown in Fig. 1B are 79% (xVg1), 76% (zVg1, Helde and Grunwald, 1993) and 73% (cVg1, Seleiro et al., 1996). The next-most-related TGF-βs in the database are substantially less related to Derrière: mouse GDF3 has 62% identity in the mature region, sea urchin Univin has 60% identity in the mature region and *Xenopus* BMP2 has 59% identity in the mature region.

The expression pattern of *derrière* suggests a role in posterior determination

In order to ask what role *derrière* might play during

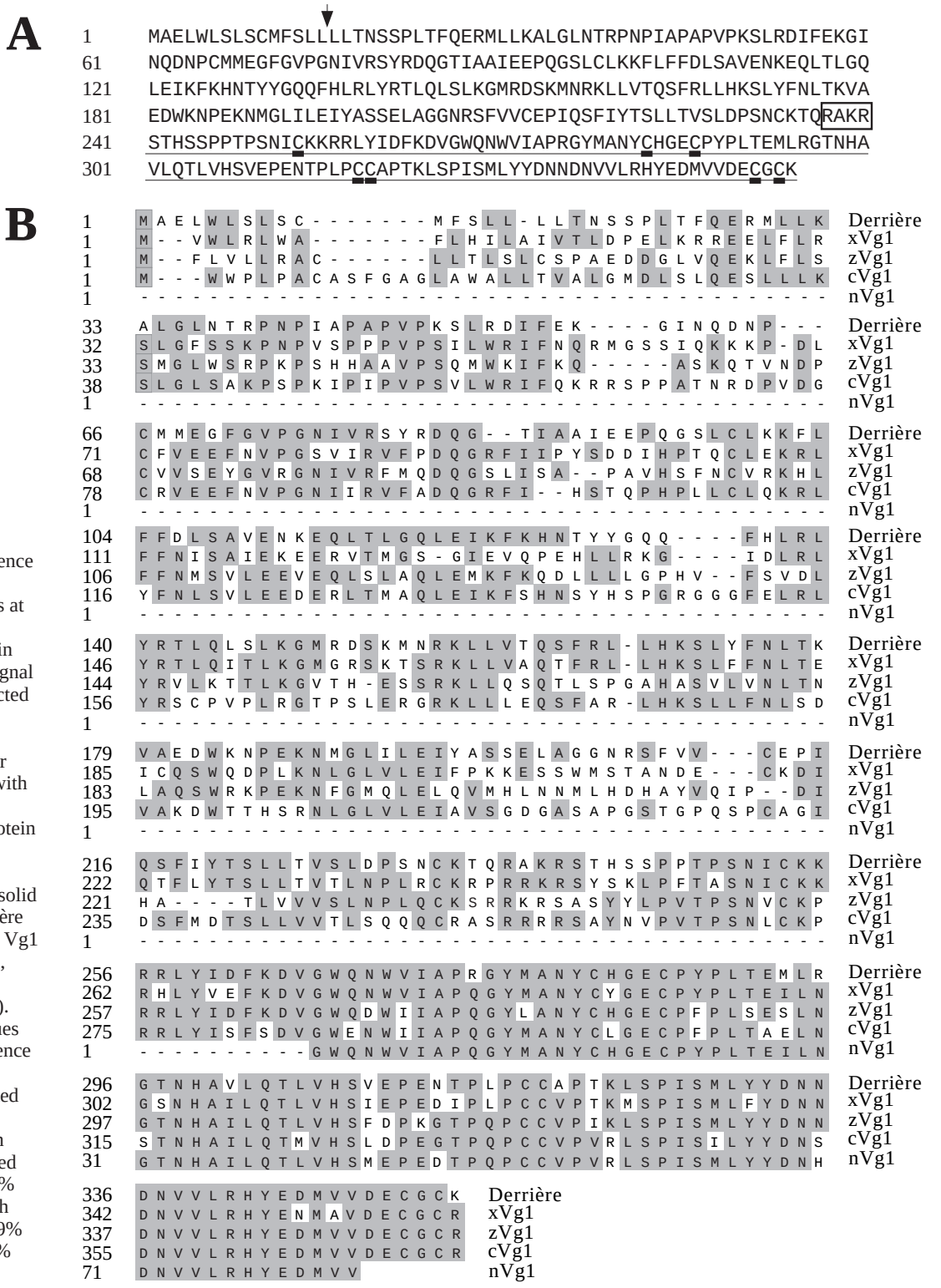

Fig. 1. Derrière protein sequence and alignments. Amino acid residues are shown. Numbers at left indicate amino acid positions. (A) Derrière protein sequence. Arrow indicates signal sequence cleavage site predicted by von Heijne algorithm. Maturation cleavage signal (RAKR) is boxed. These four amino acids are substituted with GVDG in Cm-Derrière. The mature region of Derrière protein is underlined. The seven cysteines within the mature region are underscored with solid bars. (B) Alignment of Derrière protein sequence to *Xenopus* Vg1 (Vg1), zebrafish Vg1 (zVg1), chicken Vg1 (CVg1) and incomplete newt Vg1 (nVg1). Consensus amino acid residues in proteins are shaded. Sequence gaps introduced for optimal protein alignment are indicated by dashes. The percentage identities between full-length Derrière and other Vg1-related proteins are 56% (xVg1), 51% (cVg1) and 50% (zVg1), with mature region identities of 79% (xVg1), 73% (cVg1) and 76% (zVg1).

embryogenesis, we first examined the distribution of *derrière* transcripts. Northern blotting (Fig. 2A) indicated that no maternal transcripts were present in unfertilized eggs (lane 1) and at the mid-blastula stage (lane 2). *derrière* transcripts were first detected at late blastula (stage 9, all stages according to Nieuwkoop and Faber, 1994, lane 3), peaked during gastrula (lanes 4, 5) and were barely detectable at the end of neurula (stage 19, lane 7). The *derrière* transcript was 1.5-1.6 kb in length, consistent with the size of the cDNA (1554 bp).

By whole-mount in situ hybridization (Harland, 1991) (Fig. 2B), at late blastula (stage 9.5), punctate staining was present in vegetal cells as well as in cells of the entire marginal zone (panel a). By early gastrula (stage 10.5), *derrière* RNA was present in marginal zone cells with higher intensity on the

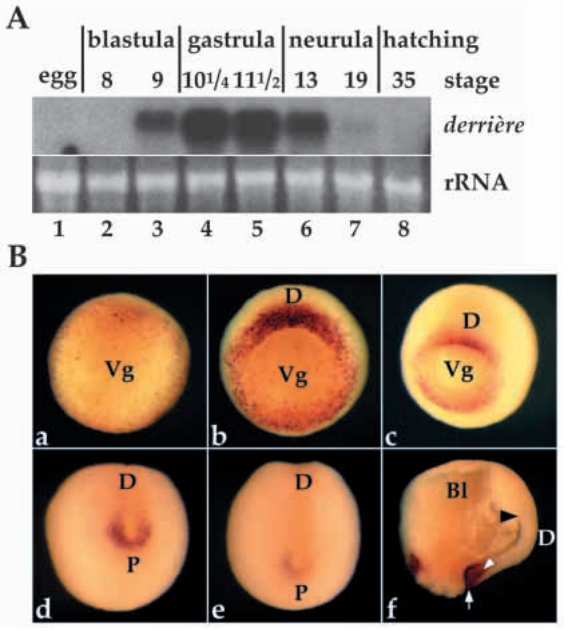

Fig. 2. Temporal and spatial expression patterns of *derrière*. (A) Northern analysis of *Xenopus* embryos. One embryo equivalent per lane was analyzed for *derrière* RNA (top row) at various embryonic stages shown. Ethidium-bromide-stained 28S rRNA is a loading control (bottom row). Lanes as marked. (B) Whole-mount in situ hybridization analysis of *derrière* expression. Embryo orientations are indicated by vegetal (Vg), dorsal (D) and posterior (P). Bl, blastocoel. Purple staining represents *derrière* expression. (a) Stage 9, late blastula; (b) stage 10.5, early gastrula; (c) stage 11.5, mid-gastrula; (d) stage 12.5, late gastrula; (e) stage 14, early neurula; (f) a sagittal section of a stage 11.5 embryo. White arrow indicates superficial layer, white arrowhead indicates deep cells and black arrowhead indicates the anterior limit of archenteron.

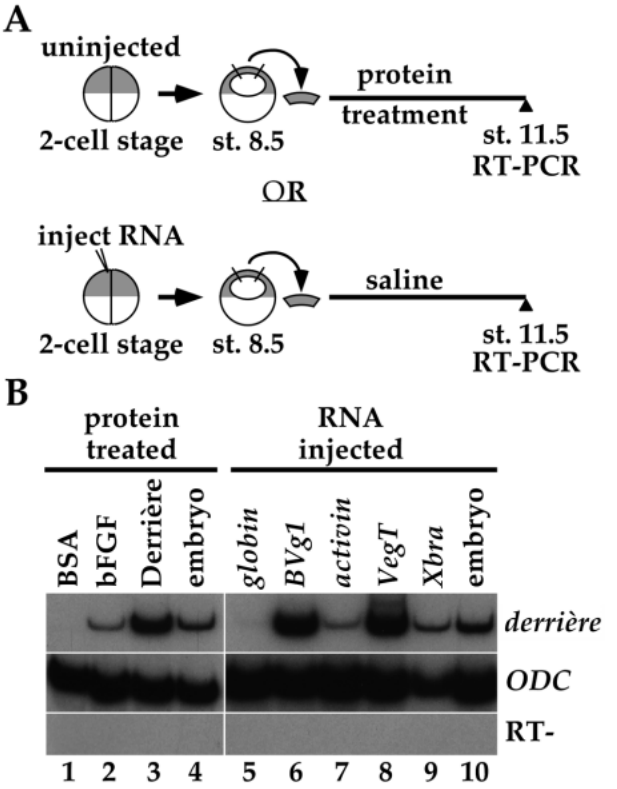

Fig. 3. *derrière* can be induced by known mesoderm inducers in animal caps. (A) Experimental scheme. Stage 8.5 animal caps of uninjected embryos were cultured with purified proteins until stage 11.5 (mid gastrula). Alternatively, embryos were injected at the animal pole of one blastomere with test RNA at the 2-cell stage. Animal caps were removed from stage 8.5 embryos and were cultured until sibling embryos reached stage 11.5. Animal caps and whole embryos were harvest for RT-PCR. (B) Induction of *derrière* in animal caps. *ODC* served as a loading control. Lanes as marked; lanes 1, 5, BSA served as a negative control; lanes 4, 10, whole embryo controls. See Materials and Methods for amount of proteins used.

dorsal side of the embryo (panel b), while by mid-gastrula (stage 11.5), RNA was posteriorly restricted with no expression visible vegetally (panel c). By late gastrula (stage 12.5, panel d) and early neurula (stage 14, panel e), staining was excluded from the dorsal midline. A sagittal section of a mid-gastrula stage embryo (panel f) showed that *derrière* transcripts were present in both superficial (white arrow) and deep (white arrowhead) mesodermal layers but absent from involuted mesodermal cells.

In summary, these data showed that *derrière* expression is zygotic, with expression initially throughout the presumptive mesendoderm and subsequent localization to the posterior mesoderm. The expression pattern of *derrière* appeared identical to that of *VegT* and suggested that *derrière* might play a role in mesodermal patterning, particularly in posterior regions of the embryo.

derrière can be induced by known mesoderm inducers

We next asked what genes controlled *derrière* expression, by testing secreted proteins known to have mesoderm-inducing capacity as well as transcription factors previously shown to be involved in mesoderm determination (Fig. 3A). Mid-blastula (stage 8.5) animal caps were isolated from uninjected embryos and incubated in purified basic fibroblast growth factor (bFGF, Promega) or Derrière protein (see Materials and Methods), with bovine serum albumin (BSA, New England Biolabs) as control. Alternately, 2-cell-stage embryos were injected in one cell with RNAs encoding BVg1 (a fusion between the mature region of Vg1 and the pro region of BMP2; Thomsen et al., 1990), Activin (Sokol et al., 1991), VegT or Xbra (Cunliffe and Smith, 1992). *globin* RNA (Krieg and Melton, 1984) was injected as a control (Fig. 3A). At mid-blastula, animal caps were removed and cultured until sibling controls reached mid-gastrula (stage 11.5) when caps were harvested using a reverse transcriptase-PCR (RT-PCR based assay). Whole embryos were used as positive controls for RT-PCR, *ornithine decarboxylase* (*ODC*) was used as loading control and reactions without addition of reverse transcriptase were included to indicate genomic DNA contamination.

The results of a representative experiment (of three experiments) are shown in Fig. 3B**.** While BSA did not activate *derrière* expression (lane 1), both bFGF (lane 2) and Derrière protein itself (lane 3) led to *derrière* activation. Of RNAs injected, *globin* as a control did not activate *derrière* expression (lane 5), *BVg1* (lane 6), *activin* (lane 7), *VegT* (lane 8) and *Xbra*

(lane 9) all led to accumulation of *derrière* RNA. These results showed that *derrière* can be induced by known mesoderm inducers and suggested that an autoregulatory loop might control *derrière* expression.

derrière acts in a dosage-dependent fashion to induce mesodermal and endodermal markers and requires FGF signaling for activity

Since several TGF-β family members are mesoderm inducers, we asked whether *derrière* could induce mesodermal markers using an animal cap assay (Fig. 4A). RNA encoding either Derrière or Globin as a control was injected into 2-cell embryos, caps were isolated at mid-blastula and harvested when control embryos reached mid-gastrula (stage 11), early neurula (stage 14) or late neurula (stage 19) to account for maximal expression of the different markers tested.

A representative experiment of at least two for each marker is shown in Fig. 4B. In comparison to *globin*-injected caps (lanes 1, 4 and 7), *derrière* was able to activate multiple mesodermal marker genes (lanes 2, 5 and 8). Genes activated included *VegT*, which has a very similar expression pattern to *derrière*, the posterior marker *eFGF* (Isaacs et al., 1992), the prechordal plate marker *gsc* (Cho et al., 1991), the mesendodermal marker *eomesodermin* (Ryan et al., 1996), the dorsal mesodermal markers *Xlim-1* (Taira et al., 1992) and *Xnot* (von Dassow et al., 1993). Additionally, *derrière* induced expression of the posterior mesodermal and ectodermal markers *HoxB9* (Sharpe et al., 1987) and an endodermal marker, *Xsox17α* (Hudson et al., 1997). *derrière* failed to activate expression of *siamois* (Lemaire et al., 1995), which is expressed in the dorsal mesendoderm, including the organizer, and did not alter expression of the ventral mesodermal marker *Xvent-1* (Gawantka et al., 1995). Additionally, *derrière* failed to induce expression of the neural-specific marker *N-CAM* (Kintner and Melton, 1987). Since expression of the epidermal marker *XK81* (Jonas et al., 1985) was strongly suppressed in *derrière*-injected caps, failure to induce neural markers was likely to be due to the almost complete conversion of the cap to mesoderm, eliminating an ectodermal substrate for neural induction.

We asked whether *derrière* induced mesodermal and endodermal markers in a dose-dependent fashion, as has been

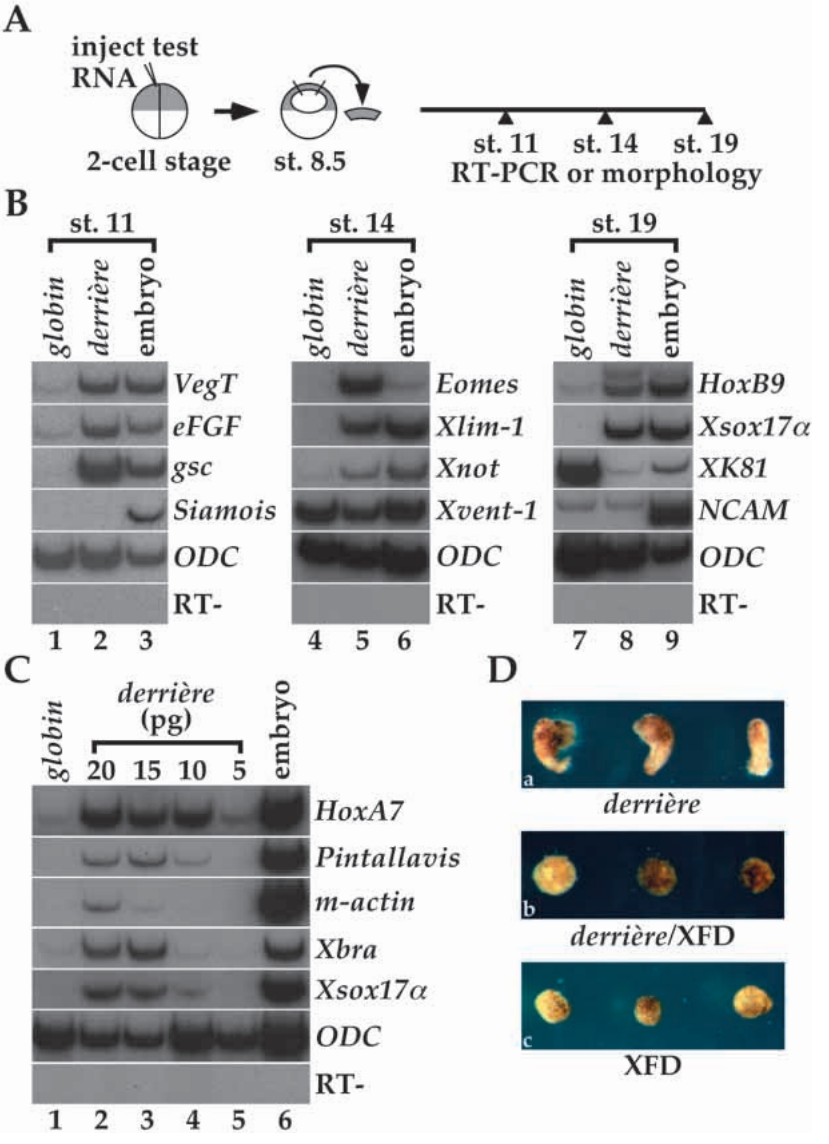

Fig. 4. *derrière* induces mesodermal and endodermal markers in animal caps. (A) Experimental scheme. Wild-type embryos were injected at the animal pole of one blastomere with test RNA at the 2-cell stage. Animal caps were removed from stage 8.5 embryos and were cultured until sibling embryos reached stages indicated by the triangles. Animal caps and whole embryos were harvest for RT-PCR or morphology. (B) Expression of marker genes (see Results) in animal caps after injection of 400 pg RNA. Injection of *globin* served as negative controls. *ODC* served as a loading control. *VegT* has a very similar expression pattern to *derrière*. *eFGF* is expressed posteriorly and in the notochord. *gsc* is a prechordal plate marker, *siamois* is expressed in the dorsal mesendoderm. *eomesodermin* a mesendodermal marker. *Xlim-1* and *Xnot* are dorsal mesodermal markers. *Xvent-1* is a ventral ectodermal and mesodermal marker. *HoxB9* marks posterior spinal cord. *Xsox17α* is an endodermal marker. *XK81* is a ventral ectodermal (epidermal) marker and *N-CAM* is a neural-specific marker. Lanes as marked. (C) Mesodermal markers respond to *derrière* induction in a dose-dependent manner. *HoxA7* is a posterior mesodermal and ectodermal marker. At the time of harvest, *Pintallavis* weakly marks the dorsal mesoderm and tailbud. *m-actin* is a muscle-specific marker and *Xbra* is expressed posteriorly and in the notochord. Lanes as marked. *β-globin*-capped RNA was used to make up for differences in amount of test RNA injected such that all embryos received the same total amount of RNA. (D) XFD blocks *derrière*-induced cap elongation. (a) 200 pg *derrière* and 800 pg *globin*; (b) 200 pg *derrière* and 800 pg XFD; (c) 800 pg XFD and 200 pg *globin*.

demonstrated for other TGF-βs, particularly *activin* (Green et al., 1992). As shown in Fig. 4C, at the highest concentration of *derrière* RNA tested (20 pg), *HoxA7* (Condie and Harland, 1987), *Pintallavis* (Ruiz i Altaba and Jessell, 1992), the muscle-specific marker *muscle actin* (*m-actin*, Mohun et al., 1984), *Xbra* (Smith et al., 1991) and *Xsox17α* were induced (lane 2), at 15 pg *HoxA7*, *Pintallavis*, *Xbra* and *Xsox17α* were induced (lane 3) while, at 10 pg, only *HoxA7* and low levels of *Pintallavis* and *Xsox17α* were induced (lane 4). None of these markers was activated at 5 pg *derrière* (lane 5). At the stage when caps were harvested, *Pintallavis* is expressed weakly in the dorsal mesoderm and in the tailbud (Ruiz i Altaba and Jessell, 1992), and the expression assayed here may be reflective of both dorsal and posterior fates.

It has previously been shown that an intact FGF signaling pathway is required for signaling by *activin* (La Bonne and Whitman, 1994). We asked whether FGF signaling is required for *derrière* function using an animal cap assay. As shown in Fig. 4D, *derrière* induced elongation of animal caps (panel a;

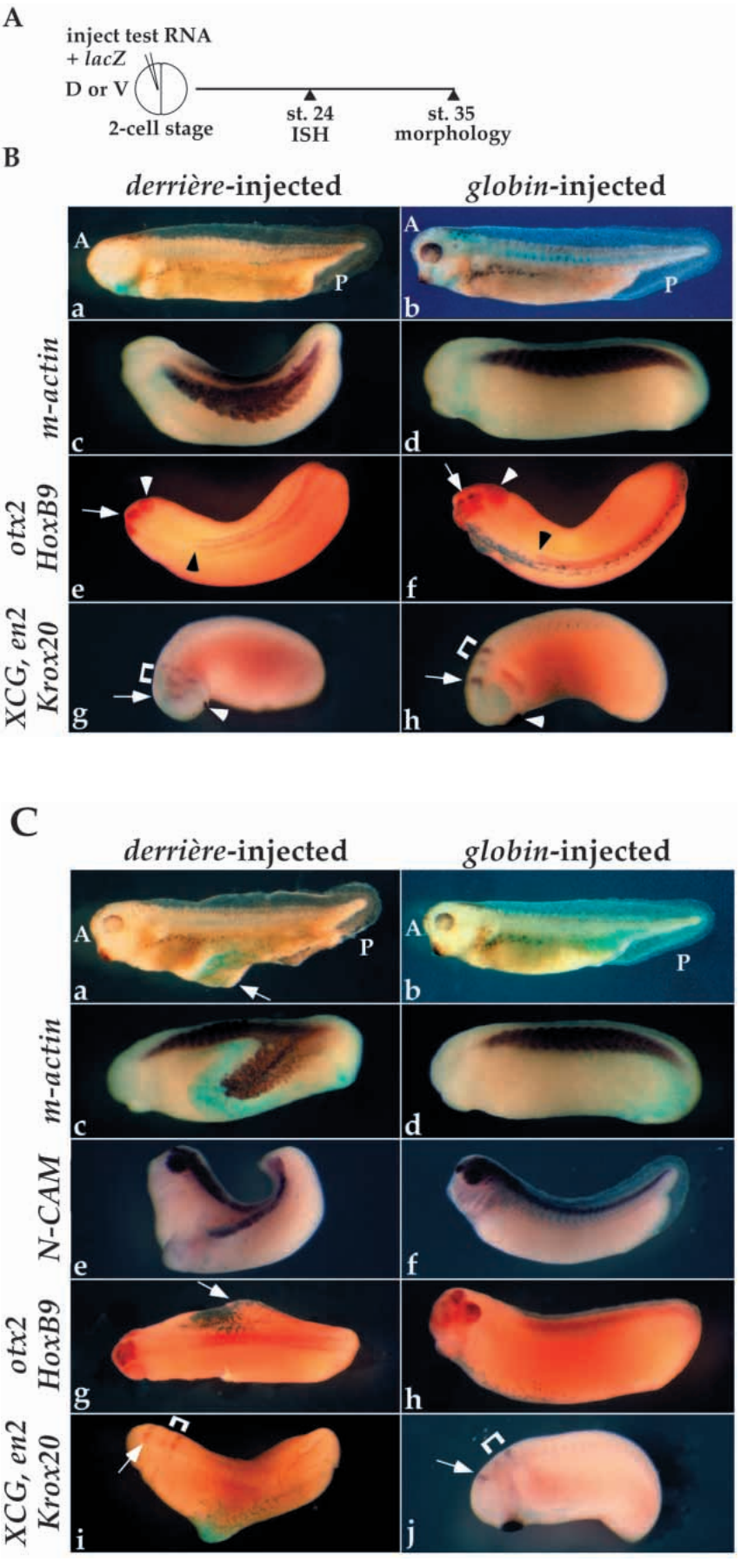

Fig. 5. *derrière* suppresses head formation or induces a posterior secondary axis in whole embryos. (A) Experimental scheme. Wild-type or albino embryos were injected with 50 pg *derrière* and 80 pg *lacZ* RNA in the marginal zone of one blastomere at 2-cell stage, either dorsally or ventrally. Albino embryos were harvested at stage 24 (tailbud) for in situ hybridization and wild-type embryo were harvested at stage 35 (hatching) for morphological analysis. (B) Dorsal misexpression of *derrière* results in microcephaly. In all panels anterior (A) is to the left and posterior (P) is to the right. Light blue indicates lineage tracer β-gal. (a,b) Wild-type embryo injected with *derrière* RNA (a) and *globin* RNA (b). (c-h) Albino embryos processed for in situ hybridization (see Results). Purple staining represents probes as indicated on the left. *m-actin* is a muscle-specific marker, *otx2* marks the forebrain, *XCG* marks the cement gland, *en-2* marks the midbrain/hindbrain junction and *Krox20* marks rhombomeres 3 and 5 in the hindbrain. (c-e) White arrow and arrowhead indicate *otx2* forebrain and eye staining, respectively. Black arrowhead indicates anterior limit of *HoxB9* staining. (f-h) White arrow indicates *en-2* staining, white arrowhead indicates *XCG* staining, bracket indicates *krox20* staining. (C) Ventral misexpression of *derrière* results in a posterior secondary axis. In all panels, anterior (A) is to the left and posterior (P) is to the right. (a,b) Wild-type embryos. White arrow indicates a secondary axis. (c-j) Albino embryos processed for in situ hybridization (see Results). Markers as for B, except for *N-CAM*, which is a general neural marker. Arrow indicates secondary axis. (i,j) Arrow indicates *en-2* staining, bracket indicates *krox20* hindbrain staining.

100%; *n*=29), and this was reduced to background levels by the presence of XFD, a dominant negative FGF receptor (Amaya et al., 1991, panel b; 6%; *n*=33, control in panel c; 0%; *n*=30).

These results showed that *derrière* could induce mesodermal markers characteristic of dorsal, lateral, anterior and posterior regions, as well as endodermal markers. This induction occurred in a dose-dependent and FGF-dependent manner. A posterior marker, *HoxA7*, was preferentially activated at lower *derrière* concentration, while *m-actin* required the highest does of *derrière*. Interestingly, an endodermal marker was induced by *derrière* within the same concentration range as mesodermal markers were induced. This is different from the activity of *BVg1* and *activin* where high concentrations induce endoderm and low concentrations induce mesoderm (Henry et al., 1996).

Misexpression of *derrière* in whole embryos leads to microcephaly or to a partial secondary axis

We next asked whether *derrière* could alter axial patterning in whole embryos, using a gain-of-function approach. 2-cell embryos were injected either dorsally or ventrally in the marginal zone with 50 pg *derrière* or *globin* RNA (Fig. 5A), along with *lacZ* RNA as lineage tracer. Higher amounts of injected *derrière* RNA led to abnormal gastrulation and death by the end of gastrulation (not shown). Embryos were harvested at tailbud (stage 24) or hatching (stage 35) for analysis by in situ hybridization or morphological examination.

Representative results are shown in Fig. 5B,C. After injection of *derrière* RNA dorsally (Fig. 5B), embryos developed with a small head (panel a; 88.4% of injected embryos, *n*=189), with reduction or absence of eyes and cement gland. Controls injected with *globin* RNA did not show these defects (panel b; 0%; *n*=73). Expression of *m-actin* in the posterior paraxial mesoderm was unperturbed after *derrière* misexpression (panel c; 0%; *n*=12) and did not extend more anteriorly than it did in *globin*-injected controls (panel d; 0%; *n*=15). *derrière* reduced expression of the anterior marker *otx2* (Blitz and Cho, 1995; Pannese et al., 1995) in forebrain and midbrain (panel e; white arrow), eyes (white arrowhead) and cement gland (100%, *n*=12) relative to *globin*-injected controls (panel f; 0%; *n*=15). *derrière* did not alter the anterior limit of *HoxB9* expression in the spinal cord (panel e; black arrowhead; compare to control in panel f). Although *derrière* did not alter the expression pattern of the midbrain marker *engrailed* (panel g; white arrow, *en-2*, Hemmati-Brivanlou et al., 1991) or the hindbrain marker *krox20* (bracket), these markers were expressed much closer to the anteriormost extent of the embryo (100%, *n*=13) than they were in controls (panel h; 0%; *n*=16). In accord with morphological data, expression of *XCG*, a cement gland marker (Sive et al., 1989), was strongly reduced in *derrière*-injected embryos (panels g and h; white arrowhead). Thus, ectopic expression of *derrière* dorsally suppressed head formation. This phenotype was very similar to the partial head suppression observed after ectopic dorsal expression of *VegT* and *eFGF* (Isaacs et al., 1994; Zhang and King, 1996).

In contrast to the effects of dorsal misexpression, ectopic expression of *derrière* ventrally (Fig. 5C) led to formation of a partial secondary axis (panel a; 71% of injected embryos, *n*=123), compared to *globin*-injected controls (panel b; 0%; *n*=34). The secondary axis never contained a head and ended posterior to the level of the otic vesicle of the primary axis. *m-actin* expression in the secondary axis indicated the presence of posterior (paraxial) mesoderm (panel c; 100%, *n*=12), which was never observed after *globin* RNA injection (panel d; 0%; *n*=15), and also expressed the neural-specific marker *N-CAM* (panel e; 67%, *n*=12; control in panel f; 0%, *n*=12). *otx2* expression was not observed in the secondary axis (panel g, white arrow; 0%; *n*=12; control in panel h; 0%; *n*=15), indicating a lack of anterior tissue, while the lack of *HoxB9* expression detected in the secondary axis (panel g), suggested that spinal cord had not formed. *en-2* or *krox20* expression was not observed in the secondary axis (panel i; 0%, *n*=14; control in panel j; 0%; *n*=16), confirming that the secondary axis did not contain tissue anterior to the hindbrain. No neural tube or notochord were apparent in sections through the secondary axis (not shown).

In summary, these data indicated that *derrière* had the properties of a posterior inducer that suppressed head formation and directed formation of a partial secondary axis, containing paraxial mesoderm and neural tissue.

derrière increases dorsal character of the ventral marginal zone

One explanation for the mechanism by which *derrière* induces a secondary axis is that the Derrière protein dorsalizes the ventral marginal zone (VMZ), as several *Xenopus* TGF-β family members are able to do (Sokol et al., 1991; Jones et al., 1995; Kessler and Melton, 1995; Smith et al., 1995; Joseph and Melton, 1997). Since *derrière* expression is maximal on the dorsal side during early gastrula, it may play a role in normal dorsal mesendoderm (organizer) activity. We tested the ability of *derrière* to increase dorsal character of the VMZ by injecting RNA into the ventral side of both ventral blastomeres at the 4-cell stage. The VMZ and control dorsal marginal zone (DMZ) were dissected at early gastrula (stage 10.25) and harvested at mid-neurula (stage 17) for RT-PCR analysis (Fig. 6A).

At the time of dissection, misexpressed *derrière* induced a secondary invagination on the ventral side of the embryo (not shown) similar to that observed after *VegT* misexpression (Lustig et al., 1996b). At the time of harvest, *globin*-injected VMZ explants remained round (0% elongation; *n*=30) while DMZ explants (100%; *n*=20) and VMZ explants expressing *derrière* (92%; *n*=24) elongated. In RT-PCR analyses (Fig. 6B), *m-actin* was used as an indicator of dorsalization while *Xbra* was a marker for mesodermal fates. While cultured DMZ explants expressed high levels of *m-actin* (lane 1), *globin*-injected control VMZ explants did not (lane 2). In contrast VMZ explants expressing *derrière* showed strong *m-actin* expression (lane 3).

These data showed that *derrière* could activate dorsal-specific fates in the ventral marginal zone and was likely to induce a secondary axis through this activity. From these experiments, is not clear whether *derrière* acts during gastrula stages to dorsalize the pre-existing VMZ or whether it acts earlier to direct formation of a partial DMZ on the ventral side of the embryo.

derrière posteriorizes ectoderm indirectly

The ability of *derrière* to suppress expression of *otx2* in the forebrain and eyes, and to reduce formation of the ectodermal

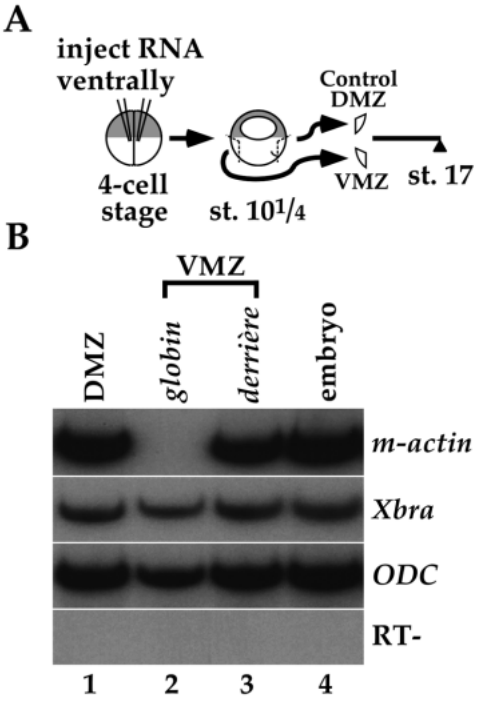

Fig. 6. *derrière* increases dorsal character of the ventral marginal zone. (A) Experimental scheme. 25 pg *derrière* RNA was injected into the marginal zone of both ventral blastomeres of 4-cell stage wild-type embryos. The VMZ was dissected at stage 10.25 and cultured until harvest for RT-PCR at stage 17. (B) Dorsalization of the VMZ by *derrière*. Lanes as marked.

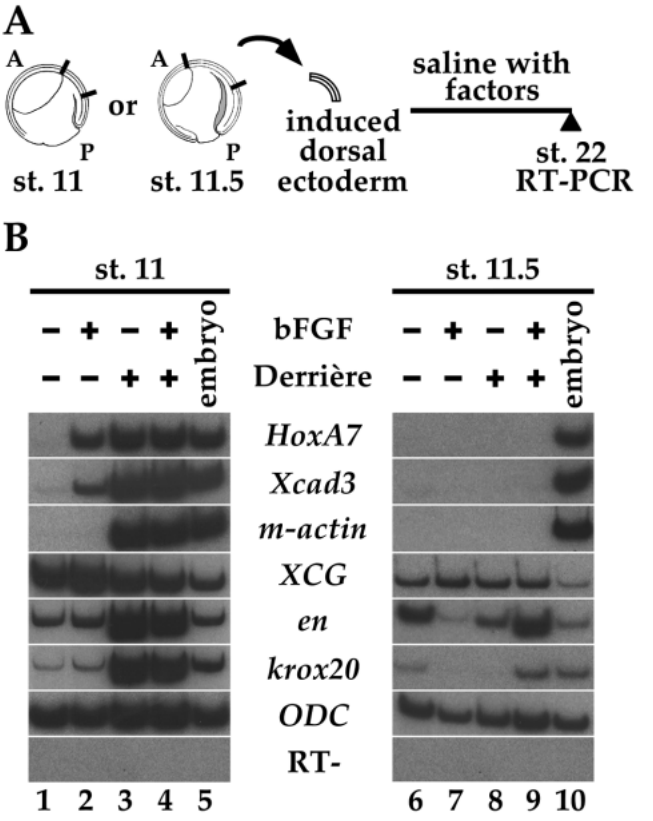

Fig. 7. Derrière posteriorizes isolated neurectoderm indirectly. (A) Experimental scheme. Anterior dorsal ectoderm (aDE, indicated by the cut marks, see Materials and Methods) was isolated from stage 11 and 11.5 (mid-gastrula) embryos. Explants were cultured in saline alone or with added factors until harvest for RT-PCR at stage 22 (tailbud). (B) Expression of marker genes in aDE explants (see Results). BSA-treated explants served as negative controls. See Materials and Methods for amount of protein used. *HoxA7* and *Xcad3* are expressed in posterior mesoderm and ectoderm, *m-actin* is a muscle marker, *XCG* is a cement gland marker, *en-2* marks the midbrain/hindbrain junction and *Krox20* is a hindbrain marker. Lanes as marked.

cement gland, suggested that *derrière* might suppress head formation by directly altering ectodermal patterning. We therefore asked whether Derrière could alter specification of the ectoderm, and compared it to the effects of FGF, a putative direct neural inducer or modifier (Kengaku and Okamoto, 1995; Lamb and Harland, 1995; Kolm and Sive, 1997). Dorsal ectoderm that we had previously shown to be specified as neurectoderm (Kolm et al., 1997) was isolated from mid-gastrula embryos and incubated in Derrière protein, bFGF or BSA as a control (Fig. 7A). In order to determine whether any effects of Derrière were secondary to mesoderm induction, we used two stages of dorsal ectoderm in these assays: one (stage 11) is still competent to respond to Activin to form mesoderm while the other (stage 11.5) is not (Green et al., 1990). Explants were harvested when control embryos reached tailbud (stage 22) for assay by RT-PCR.

A representative experiment (of five) is shown in Fig. 7B. Stage 11 dorsal ectoderm failed to express the posterior markers *HoxA7* and *Xcad3* (Northrop and Kimelman, 1994) which are expressed in both mesoderm and ectoderm, or the paraxial mesodermal marker *m-actin* (lane 1), but did express the anterior cement gland marker *XCG*, the midbrain marker *en-2* and the hindbrain marker *krox20*. bFGF induced expression of *HoxA7* and *Xcad3* (lane 2), but did not induce *m-actin*, and did not alter expression of *XCG*, *en-2* and *krox20*. In contrast, Derrière protein activated expression of *HoxA7*, *Xcad3* and *m-actin* and increased expression of the posterior neural markers *en-2* and *krox20* (lane 3). The combination of bFGF and Derrière gave results indistinguishable from those of Derrière alone (lane 4). These data indicated that Derrière could induce mesoderm and concomitantly induce expression of posterior neural markers in anterior neurectoderm.

Ectodermal explants of a slightly later stage (stage 11.5) expressed *XCG*, *en-2* and *krox20* after culture, but failed to express *HoxA7*, *Xcad3* or *m-actin* (lane 6). bFGF did not induce any posterior markers, had no effect on *XCG* expression and slightly decreased both *en-2* and *krox20* expression (lane 7). At this stage, Derrière could no longer induce expression of *m-actin*, *HoxA7* or *Xcad3* (lane 8). Derrière did not alter expression of *XCG*, and slightly reduced *en-2* and *krox20* expression. In contrast to the effects of single factor treatments, treatment of stage 11.5 ectoderm with Derrière plus bFGF did not lead to a decrease in *en-2* and *krox20* expression (lane 9).

We conclude that Derrière can activate posterior neural-specific marker gene expression, but only by inducing mesoderm in competent tissue that is present in stage 11 neurectoderm, but absent from stage 11.5 neurectoderm. In this assay, bFGF could also not directly alter A/P neurectodermal patterning. The data suggest that, in the whole embryo,

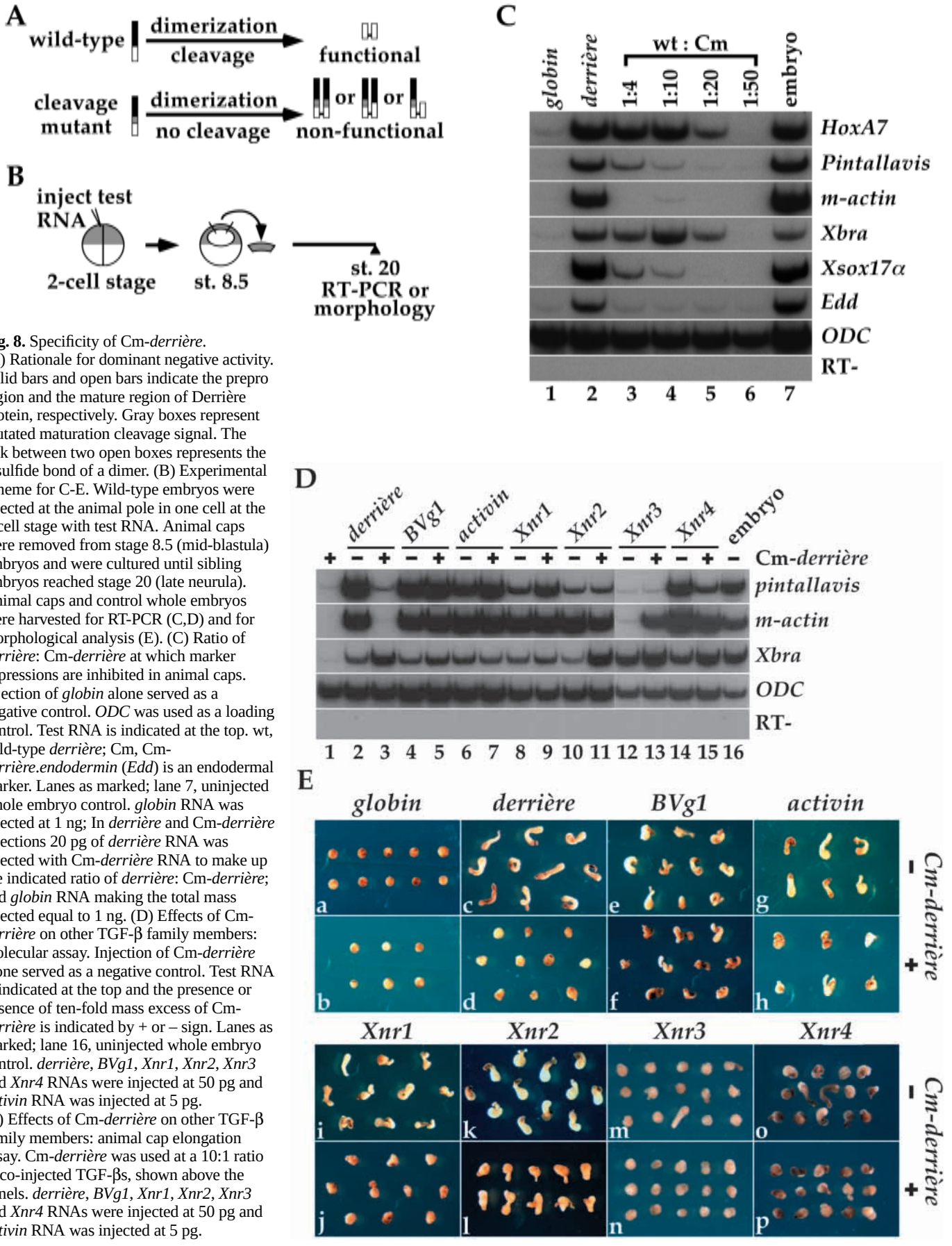

Fig. 8. Specificity of Cm-*derrière*. (A) Rationale for dominant negative activity. Solid bars and open bars indicate the prepro region and the mature region of Derrière protein, respectively. Gray boxes represent mutated maturation cleavage signal. The link between two open boxes represents the disulfide bond of a dimer. (B) Experimental scheme for C-E. Wild-type embryos were injected at the animal pole in one cell at the 2-cell stage with test RNA. Animal caps were removed from stage 8.5 (mid-blastula) embryos and were cultured until sibling embryos reached stage 20 (late neurula). Animal caps and control whole embryos were harvested for RT-PCR (C,D) and for morphological analysis (E). (C) Ratio of *derrière*: Cm-*derrière* at which marker expressions are inhibited in animal caps. Injection of *globin* alone served as a negative control. *ODC* was used as a loading control. Test RNA is indicated at the top. wt, wild-type *derrière*; Cm, Cm-*derrière.endodermin* (*Edd*) is an endodermal marker. Lanes as marked; lane 7, uninjected whole embryo control. *globin* RNA was injected at 1 ng; In *derrière* and Cm-*derrière* injections 20 pg of *derrière* RNA was injected with Cm-*derrière* RNA to make up the indicated ratio of *derrière*: Cm-*derrière*; and *globin* RNA making the total mass injected equal to 1 ng. (D) Effects of Cm-*derrière* on other TGF-β family members: molecular assay. Injection of Cm-*derrière* alone served as a negative control. Test RNA is indicated at the top and the presence or absence of ten-fold mass excess of Cm-*derrière* is indicated by + or – sign. Lanes as marked; lane 16, uninjected whole embryo control. *derrière*, *BVg1*, *Xnr1*, *Xnr2*, *Xnr3* and *Xnr4* RNAs were injected at 50 pg and *activin* RNA was injected at 5 pg. (E) Effects of Cm-*derrière* on other TGF-β family members: animal cap elongation assay. Cm-*derrière* was used at a 10:1 ratio to co-injected TGF-βs, shown above the panels. *derrière*, *BVg1*, *Xnr1*, *Xnr2*, *Xnr3* and *Xnr4* RNAs were injected at 50 pg and *activin* RNA was injected at 5 pg.

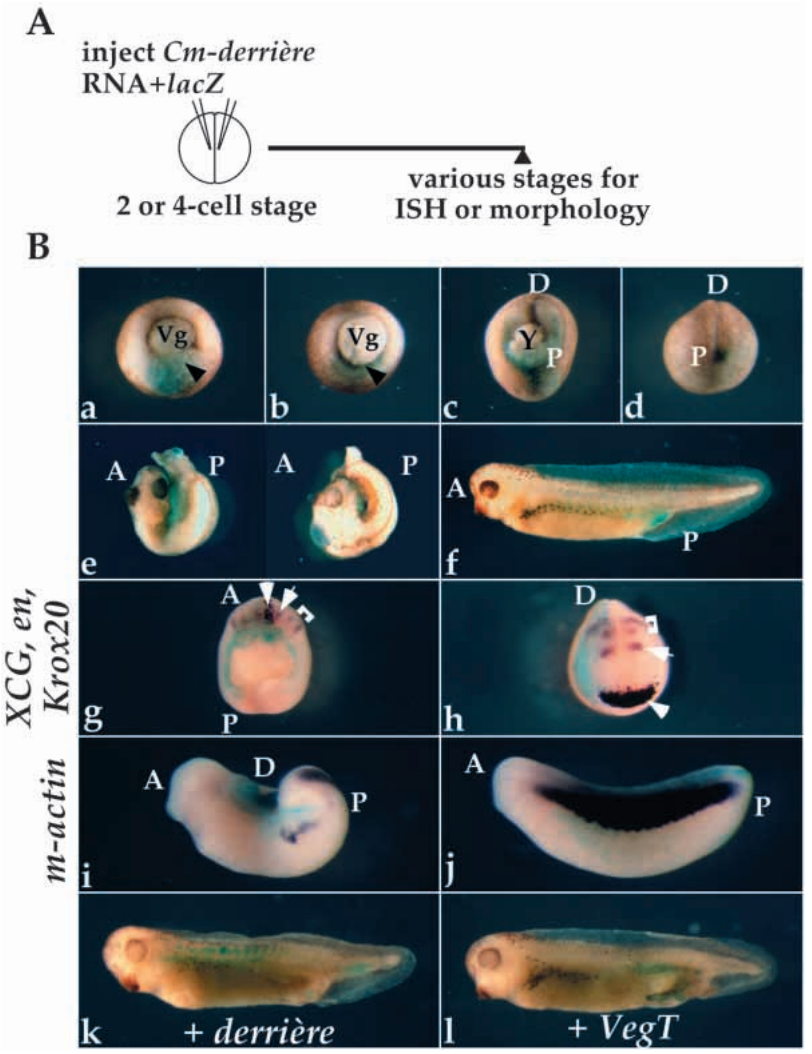

Fig. 9. Phenotype and in situ hybridization analysis of Cm-*derrière*-injected embryos and rescue of Cm-*derrière* by wild-type *derrière* and *VegT*. (A) Experimental scheme. Wild-type or albino embryos were injected with test and *lacZ* RNA in the marginal zone. At stages indicated below, albino embryos were harvested for in situ hybridization and wild-type embryo were harvested for morphological analysis. (B) Effects of Cm-*derrière* expression on whole embryos. Light blue indicates lineage tracer β-gal and purple represents specific RNA expression. (a,b) Vegetal (Vg) views of stage 11-11.5 embryos injected at 4-cell stage with 250 pg Cm-*derrière* (a) and *globin* (b) into each of the two ventral blastomeres. Black arrowhead: extent of blastopore formation. (c,d) Posterior (P) views of stage 17 embryos injected at 4-cell stage with 250 pg Cm-*derrière* (c) and *globin* (d) into each of the two ventral blastomeres. D, dorsal; Y, yolk cells. (e,f) Lateral views of stage 35 embryos injected at 2-cell stage with 500 pg Cm-*derrière* (e) and *globin* (f) into both blastomeres. A, anterior; P, posterior. (g-j) Albino embryos processed for in situ hybridization. (g) *XCG* (arrowhead) *en* (arrow) and *krox20* (bracket) probes; dorsal view of a stage 20 embryo injected with 500 pg Cm-*derrière*. (h) *XCG*, *en* and *krox20* probes; head-on view of a stage 20 embryo injected with 500 pg *globin*. (i,j) *m-actin* probe; lateral views of stage 26 embryos injected with 500 pg Cm-*derrière* (i) and *globin* (j). (k,l) Rescue experiments. (k) 50 pg *derrière* and 500 pg Cm-*derrière* co-injected; (l) 50 pg *VegT* and 500 pg Cm-*derrière* co-injected.

derrière suppresses head formation by altering mesodermal patterning.

A *derrière* cleavage mutant preferentially interferes with *derrière* activity

In order to ablate *derrière* function, we constructed a dominant interfering mutant of *derrière* (Cm-*derrière* for cleavage mutant). In this mutant residues, 237-240 are changed from RAKR to GVDG (Fig. 1A and Materials and Methods), which we anticipated would block cleavage of the Derrière pro-protein to the mature form. Since TGF-βs are cleaved after dimerization, a non-cleavable monomer can prevent cleavage of normal monomers and thereby act as a dominant negative protein (Fig. 8A), a strategy that has been successfully used to ablate BMP function (Hawley et al., 1995).

Initially we asked whether Cm-*derrière* would inhibit induction of gene expression by *derrière*, using different ratios of *derrière*:Cm-*derrière* in an animal cap assay (Fig. 8B). Relative to *globin*-injected caps (Fig. 8C, lane 1), *derrière* induced high levels of *HoxA7*, *Pintallavis*, *m-actin*, *Xbra*, *Xsox17α* and *endodermin* (*Edd*, Sasai et al., 1996, lane 2). At a ratio of 1:4 wild-type:Cm-*derrière*, expression of *m-actin* and *Edd* was ablated (lane 3). At a ratio of 1:10, expression of *Pintallavis* and *Xsox17α* was strongly reduced (lane 4). At 1:20, *HoxA7* and *Xbra* expression was reduced (lane 5) and at 1:50, expression of all six genes was abolished (lane 6). These data are consistent with the dose-response to *derrière* (Fig. 3C) showing that *m-actin* is the most sensitive and *HoxA7* the least sensitive to the level of active Derrière protein. These data showed that Cm-*derrière* was an effective inhibitor of *derrière* activity.

We next used the animal cap assay to ask whether the effects of Cm-*derrière* were specific for *derrière* or whether it also inhibited other TGF-β family members (Fig. 8B). RNAs encoding various TGF-β family members were injected into 2-cell embryos either alone or with Cm-*derrière* in ten-fold mass excess. A representative experiment (of at least two) is shown in Fig. 8D. Cm-*derrière* alone did not activate expression of the markers tested (lane 1), and while *derrière* strongly activated *pintallavis*, *m-actin* and *Xbra* (lane 2), co-injection of *derrière* with Cm-*derrière* suppressed *m-actin* and *Pintallavis* gene expression (lane 3). In contrast, Cm-*derrière* did not attenuate the ability of other TGF-β family members tested to induce *Pintallavis*, *m-actin* and *Xbra*. Inducers tested were *BVg1* (lanes 4 and 5), *activin* (lanes 6 and 7), *Xnr1* (lanes 8 and 9), *Xnr2* (lanes 10 and 11), *Xnr3* (lanes 12 and 13) and *Xnr4* (lanes 14 and 15). In contrast to published data (Smith et

al., 1995; Hansen et al., 1997), we found that *Xnr3* induced *Xbra* (lane 12). It is also interesting that the expression of some mesodermal markers was increased in the presence of a test TGF-β and Cm-*derrière-Xbra* in the cases of *derrière* (lanes 2 and 3) and *Xnr2* (lanes 10 and 11), and *m-actin* in the case of *Xnr3* (lanes 12 and 13). We do not presently understand the mechanism of this up-regulation.

We also tested the ability of Cm-*derrière* to interfere with animal cap elongation which is characteristic of dorsal mesodermal fates (Fig. 8E). Animal caps removed from embryos injected with *globin* did not elongate (panel a; 0% of injected embryos, *n*=34) and no change in morphology was observed after co-injection of Cm-*derrière* and *globin* (panel b; 0%, *n*=45). After *derrière* injection, 100% of caps elongated (panel c; *n*=58), and elongation was almost completely blocked after co-injection of *derrière* and Cm-*derrière* at a 1:10 ratio (panel d; 2%, *n*=64). The frequency of cap elongation induced by *BVg1* (panel e; 97%, *n*=59) was not altered by Cm-*derrière* (panel f; 97%, *n*=59). The frequency of cap elongation induced by *activin* (panel g; 100%, *n*=18) was also unaffected by addition of Cm-*derrière* (panel h; 97%, *n*=29) although the extent of elongation was slightly reduced. *Xnr1*-induced cap elongation (panel i; 100%, *n*=58) was somewhat inhibited by Cm-*derrière* (panel j; 70%, *n*=59), while both the frequency and extent of elongation induced by *Xnr2* (panel k; 100%, *n*=59) was only slightly decreased by co-expression of Cm-*derrière* (panel l; 93%, *n*=59). The frequency of *Xnr3*-induced cap elongation was low (panel m; 9%, *n*=45) and no elongation was observed after co-injection of Cm-*derrière* (panel n; 0%, *n*=45). The frequency of cap elongation induced by *Xnr4* (panel o; 96%, *n*=45) was not affected by Cm-*derrière* co-expression, but the extent of elongation was reduced (panel p; 91%, *n*=45).

In summary, these data showed that Cm-*derrière* severely attenuates the activity of *derrière* in both molecular and morphological assays. The activity of Cm-Derrière was highly preferential for Derrière protein as compared to other members of the TGF-β family, with no interference seen in a molecular assay, and only slight interference for *Xnr1* seen in a cap elongation assay. These data indicated that Cm-*derrière* was a useful reagent with which to analyze the in vivo function of the Derrière protein.

Cm-*derrière* prevents posterior formation in whole embryos

We next used the Cm-*derrière* construct to analyze the effect of ablating Derrière activity in the whole embryo. Wild-type and albino embryos were injected with Cm-*derrière* RNA or with control *globin* RNA, along with *lacZ* RNA as lineage tracer (Fig. 9A). Embryos were harvested at various stages either for in situ hybridization or for morphological analysis.

Fig. 9B shows representative embryos after Cm-*derrière* injection. During mid-gastrula stages, a blastopore failed to form where Cm-*derrière* was expressed (panel a, black arrowhead; 91%; *n*=56, compared to *globin*-injected, panel b; 0%; *n*=38), suggesting that involution had failed in this region. During neurula stages, the blastopore remained open in Cm-*derrière*-injected embryos (panel c; 100%; *n*=45, compared to *globin*-injected, panel d; 0%, *n*=32). Embryos injected with Cm-*derrière* failed to develop normal posterior morphology (panel e; 92% of injected embryos, *n*=131). While the head appeared largely normal even with lineage tracer in the head region, with eyes and cement gland visible, no somites were apparent, the neural tube remained open (as a result of the open blastopore) and no tail developed (compare to *globin*-injected control of equivalent age; panel f; 0%, *n*=129). In order to determine the anterior extent of Cm-*derrière* effects, we examined expression of *XCG*, *en* and *krox20*. As shown in panel g, all three genes were still expressed anterior to the open neural plate, although their patterning was somewhat disrupted (100%; *n*=12, compared to control in panel h; 0%; *n*=12). However, *m-actin* expression was almost completely abolished on the injected side of the embryo (panel i; 100%, *n*=27) compared to *globin*-injected controls (panel j; 0%, *n*=10), consistent with a role for *derrière* in posterior mesoderm induction. This phenotype looked similar to that observed after injection of a dominant negative *FGF* receptor (Amaya et al., 1991; Kroll and Amaya, 1996), dominant negative *Xbra* (Conlon et al., 1996) and *Brat* (*VegT*) (Horb and Thomsen, 1997) constructs. In support of the specificity of the Cm-*derrière* phenotype, we found that a dominant negative Activin ligand, Cm-Activin (Hawley et al., 1995), suppressed head formation but allowed normal posterior development (not shown). This head suppression phenotype was consistent with a results reported after expressing a dominant negative Activin receptor (Dyson and Gurdon, 1997).

In order to control for non-specific effects of Cm-*derrière*, we asked whether the truncated posterior phenotype could be rescued by native *derrière* and found that it could, when a ratio of 1:10 *derrière*:Cm-*derrière* was co-injected (panel k; 83%, *n*=77). Interestingly, the rescued embryos looked completely normal and did not display either the microcephaly or secondary axis observed after injection of *derrière* alone. Additionally, since *derrière* and *VegT* have almost identical expression patterns, we asked whether the Cm-*derrière* phenotype could be rescued by co-injection of *VegT* and found that it could (panel l; 77%, *n*=194).

In summary, these data showed that, in whole embryos, Cm-*derrière* prevents normal posterior development. The phenotype caused by Cm-*derrière* includes failure of blastopore formation and closure, likely reflecting a failure of normal involution and leading to an open region where the neural tube should have been. No tail formation was observed. Additionally, paraxial mesoderm development was severely reduced as indicated by somite morphology and muscle gene expression. This phenotype could be rescued by co-injection of either wild-type *derrière* or *VegT* RNAs, suggesting that a regulatory interaction exists between *derrière* and *VegT*.

DISCUSSION

In this report, we describe *derrière*, a zygotically expressed TGF-β family member that is closely related to *Vg1*. *derrière* appears to be induced as part of a regulatory loop involving the T box gene *VegT*. Both gain-of-function and loss-of-function assays indicate that *derrière* plays a pivotal role in posterior development of *Xenopus*.

The relationship between *derrière* and other *Vg1* family members

derrière is a new member of the *Vg* family of genes that encode TGF-β molecules (Kingsley, 1994), most similar to *Xenopus*

Vg1. Other members of this family have been found in chick (*cVg1*) and zebrafish (*zVg1*). All *Vg* family members share the ability to induce mesoderm and a secondary axis (Seleiro et al., 1996; Shah et al., 1997). Interestingly, *cVg1*, like *derrière*, is expressed posteriorly, suggesting that the function of *derrière* and *cVg1* may be similar. In contrast, *zVg1* expression is maternal and ubiquitous, although, since the protein persists through gastrulation, *zVg1* may have a later function (Helde and Grunwald, 1993; Dohrmann et al., 1996).

The expression of *Xenopus Vg1* RNA suggests a role for this gene in mesoderm and endoderm induction, however, the Vg1 proprotein is inefficiently processed and native *Vg1* RNA displays no activity when overexpressed (Dale et al., 1993). The mesoderm-inducing and axis-duplication activity of *derrière* is similar to that of BVg1 (Dale et al., 1993; Thomsen and Melton, 1993), raising the possibility that *BVg1* may partially phenocopy the effects of ectopic *derrière*. However, several results suggest that *derrière* and *Vg1* have different activities. First, unlike *derrière*, *BVg1* does not suppress head formation (Dale et al., 1993) and, second, lineage tracer co-injected with *BVg1* is found exclusively in the endoderm, consistent with the observation that high concentrations of BVg1 induced endoderm and lower concentrations induced mesoderm (Thomsen and Melton, 1993; Henry et al., 1996). We find that lineage tracer co-injected with *derrière* is found in both mesoderm and endoderm, and consistently, *derrière* induces mesodermal and endodermal markers at similar concentrations. Third, dominant negative ligands made to the mature region of Vg1 (Joseph and Melton, 1998) ventralize the embryo, distinct from the posterior suppression that we observed with Cm-*derrière*.

derrière activity is compatible only with posterolateral fates

Gain-of-function assays in the whole embryo suggest that *derrière* is an inducer of posterolateral fate. This suggestion is supported by the suppression of head formation that is likely to be a consequence of ectopic *derrière* expression in the deep marginal zone cells that constitute the head organizer, from which *derrière* expression is excluded by midgastrula. Head reduction is accompanied by a decrease in *otx2* expression and concomitant repositioning of *en-2* expression to the front of the embryo, suggesting a reprogramming of forebrain to more posterior regions. *derrière* is not able to induce head formation when misexpressed ventrally, further supporting its inability to induce head regions.

The secondary axis induced by overexpression of *derrière* in the ventral mesoderm also lacked axial tissues, including notochord (not shown) and spinal cord. However, we observed extensive muscle formation, a tissue derived from the posterolateral (paraxial) mesoderm. Neural tissue was also induced, presumably by paraxial mesoderm which is a neural inducer (Jones and Woodland, 1989). Why is only posterolateral tissue induced in the whole embryo, when *derrière* is able to activate expression of both anterior and posterior axial genes in animal caps? One possibility is that posteriorizing factors present in the embryo, but not in the animal cap, limit *derrière* activity.

derrière alters axial patterning by acting through the mesoderm

Many of the effects of *derrière* on the whole embryo are likely to be through its activity as a mesoderm inducer or modifier. Consistent with this proposal, head suppression and secondary axis formation were affected only when *derrière* was misexpressed in the marginal zone. Further, *derrière* was able to activate dorsal fates in the ventral marginal zone, presumably directing formation of a second organizer, albeit one that lacked head- and notochord-inducing activity.

The ability of ectopic *derrière* to reduce expression of the anterior neural marker *otx2* and to decrease cement gland formation in whole embryos, as well as to induce expression of the neural marker *N-CAM* in secondary axes suggested that *derrière* may directly pattern the neurectoderm. In support of a potential role in neural patterning, *derrière* is expressed beneath the neurectoderm as A/P patterning is taking place. However, *derrière* does not act directly on induced neurectoderm. In mid-gastrula neurectodermal explants, *derrière* strongly increased expression of both a midbrain and hindbrain marker, but only when this tissue could still be induced to form mesoderm. The effects of *derrière* on neurectodermal patterning are therefore likely to be mediated by modifying the mesoderm responsible for neural patterning. We note, however, that, under similar assay conditions, FGF is unable to posteriorize neurectoderm, whereas under other assay conditions it can (Kengaku and Okamoto, 1995; Lamb and Harland, 1995). The posteriorization activity of *derrière* may therefore depend on the particular experimental condition.

derrière may be induced as part of a regulatory loop with *VegT* and *FGF*

The expression pattern of *derrière* appears identical to the zygotic expression of the T box gene, *VegT*. Several lines of evidence suggest that *derrière* and *VegT* form a regulatory loop. *VegT* is able to induce *derrière* expression in animal caps and, reciprocally, *derrière* induces *VegT* expression. Both *derrière* and *VegT* give similar phenotypes in gain-of-function assays, while a dominant negative Derrière protein, Cm-Derrière, gives a phenotype that appears to be similar to that of a *VegT* dominant negative (Brat-En^R, Horb and Thomsen, 1997). Consistently, *VegT* is able to rescue the effects of Cm-Derrière protein. Maternal expression of *VegT* is required for endoderm formation (Zhang et al., 1998), while TGF-β signaling is required for normal endoderm and mesoderm formation (Kimelman and Griffin, 1998). *derrière* is a good candidate to be an endogenous TGF-β directly activated by maternal *VegT*. Later, *derrière* may be required for zygotic *VegT* expression.

In addition to *VegT*, the *eFGF*, *FGF9* (Song and Slack, 1996) and part of the *Xbra* expression domains overlap with that of *derrière*. Since *derrière* can induce *eFGF* in animal caps, and since both *VegT* and *Xbra* can also be induced by *FGF* (Isaacs et al., 1994; Schulte-Merker and Smith, 1995; Lustig et al., 1996b; Horb and Thomsen, 1997), the data suggest that *derrière* is part of an FGF-dependent regulatory loop, perhaps acting through *VegT*.

A requirement for *derrière* in formation of posterior regions

Cm-Derrière preferentially inhibited Derrière activity relative to a panel of other TGF-βs tested. The ability of Cm-*derrière* to decrease *Xnr1* activity in an animal cap elongation assay indicates that Derrière and Xnr1 may form active heterodimers, or that *Xnr1* directs elongation by inducing *derrière*. The

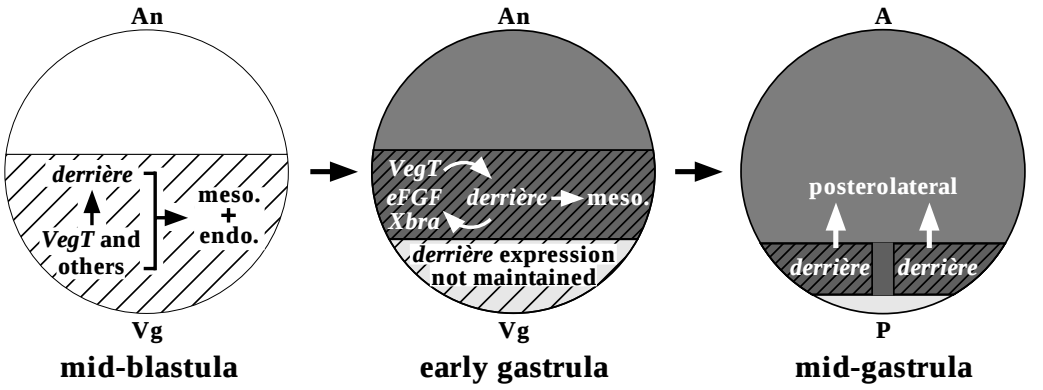

Fig. 10. Model for *derrière* activity. At the mid-blastula transition, we speculate that maternal transcription factors (such as *VegT*) and possibly secreted factors activate *derrière* expression in the presumptive mesoderm and endoderm (wide hatching). During early gastrula, *derrière* expression is maintained in the mesoderm by a positive feed-back loop that includes *FGFs* (such as *eFGF*), *Xbra* and zygotic *VegT* (close hatching). *derrière* expression in the endoderm is not maintained presumably because such a feed-back loop cannot be established there. During early gastrula stages, *derrière* may activate mesendodermal fates in both the future head region and more posteriorly. In particular, *derrière* may play a role in activating posterolateral (paraxial) mesodermal fates at this time, before somite formation. By mid to late gastrula, *derrière* is excluded from anterior mesoderm and from the dorsal midline (close hatching), and continues to promote posterolateral fates. An, animal pole; Vg, vegetal pole; A, anterior; P, posterior; meso, mesodermal fates; endo, endodermal fates.

phenotype of Cm-*derrière*-expressing embryos consists of a normal head with a severely reduced trunk and tail. This is entirely different from that of embryos expressing Cm-*activin* (not shown) or a dominant negative *activin* receptor (Dyson and Gurdon, 1997) where head suppression is observed. The Cm-*derrière* phenotype resembles that of embryos expressing a dominant negative FGF receptor (Amaya et al., 1991; Kroll and Amaya, 1996), a dominant negative Xbra protein (Conlon et al., 1996) or a dominant negative Brat protein (Horb and Thomsen, 1997). However, since unlike *derrière*, *FGF* cannot direct formation of a secondary axis, *derrière* must have a unique activity that is not shared with *FGF*. In addition to FGF signaling, posterior determination in *Xenopus* appears to involve the *wnt* and retinoid pathways (Blumberg et al., 1997; Kolm et al., 1997; McGrew et al., 1997). It is not yet clear how the TGF-β pathway and these pathways interact.

Why has the requirement for TGF-β signaling in formation of posterior structures previously been missed? One possibility is that Derrière is the only TGF-β ligand specifically required for posterior determination and this requirement could only have been picked up by specifically inhibiting *derrière*. Since a general inhibition of TGF-β signaling in *Xenopus* prevents all mesoderm formation, resulting in anaxial embryos (Hemmati-Brivanlou and Melton, 1992), a later posterior requirement for TGF-β signaling would have been masked. Second, Derrière signaling appears to require an intact FGF signaling pathway as has been demonstrated for Activin (Cornell and Kimelman, 1994; La Bonne and Whitman, 1994). Thus, since FGF signaling is required for posterior tissue formation, in the absence of an active FGF pathway, the Derrière pathway might also not be active.

A model for *derrière* function

Our data suggest a model for *derrière* function (Fig. 10). Maternal *VegT* activates expression of *derrière* soon after the mid-blastula transition. Soon after this, a regulatory loop is established between *derrière*, zygotically expressed *VegT*, *eFGF*/*FGF9* and possibly *Xbra*. This loop maintains expression of *derrière* in the marginal zone, but fails to maintain expression vegetally, perhaps because of a lack of vegetal FGF signaling (Cornell et al., 1995). Dorsally, *derrière* expression is highest due to the activity of dorsal-specific factors. At early gastrula, Derrière, along with other factors, may induce a dorsoventral array of mesodermal genes in the marginal zone. As *derrière* expression becomes posteriorly restricted during gastrulation, it may specifically activate genes expressed in the this region of the embryo including those later determining paraxial mesoderm (muscle). Since *derrière* RNA is not expressed in the somites, it must either activate muscle formation during gastrulation or Derrière protein must persist after its RNA has disappeared. We speculate that *derrière* acts in conjunction with FGF signaling to promote formation of posterior regions, through downstream transcription factors such as Xbra and VegT, that can be activated by both *derrière* and *FGF*. However, *derrière* must also have a unique activity, likely involving the activation of other factors, to account for its ability to direct secondary axis formation where *FGF* cannot.

In summary, our results indicate that posterior development in *Xenopus* requires activity of the TGF-β family member, Derrière. Future directions include analyzing the regulatory loop between *FGF*, *VegT* and *derrière* suggested by these studies, and addressing further the mechanism by which *derrière* regulates posterior patterning.

We thank Marc Kirschner, Doug Melton, Mary Lou King, Chris Wright, Bill Smith and Jeremy Green for gifts of plasmids. Special thanks to Jen Stover for assisting the yeast selection, to Cheryl Evans and Matt Benasutti for sequencing support, to Chris Dagdigian for help with bioinformatics and to Monique Davies for technical support at Genetics Institute; to Vladimir Apekin for expert frog care, to Jeanne Sweeney-Reis for sections, to Ross Silburn for technical assistance and to Liuda Ziaugra for sequencing support at the Whitehead Institute. We are grateful to Tony Celeste, Peggy Kolm and members of our laboratories for helpful discussions. B. I. S. was supported by a NIH postdoctoral fellowship, and by a fellowship from Genetics Institute. H. L. S. was the Latham Family Career

Development Professor at MIT, and a recipient of a NSF Young Investigators Award. This work is supported by the NSF, Genetics Institute and the Human Frontiers Science Foundation Program.

REFERENCES

Amaya, E., Musci, T. J. and Kirschner, M. W. (1991). Expression of a dominant negative mutant of the FGF receptor disrupts mesoderm formation in Xenopus embryos. *Cell* **66**, 257-270.

Blitz, I. L. and Cho, K. W. (1995). Anterior neurectoderm is progressively induced during gastrulation: the role of the Xenopus homeobox gene orthodenticle. *Development* **121**, 993-1004.

Blumberg, B., Bolado, J., Moreno, T., Kintner, C., Evans, R. and Papalopulu, N. (1997). An essential role for retinoid signaling in anteroposterior neural patterning. *Development* **124**, 373-379.

Bradley, L., Wainstock, D. and Sive, H. (1996). Positive and negative signals modulate formation of the *Xenopus* cement gland. *Developement* **122**, 2739-2750.

Cho, K. W., Morita, E. A., Wright, C. V. and De Robertis, E. M. (1991). Overexpression of a homeodomain protein confers axis-forming activity to uncommitted Xenopus embryonic cells. *Cell* **65**, 55-64.

Condie, B. G. and Harland, R. M. (1987). Posterior expression of a homeobox gene in early Xenopus embryos. *Development* **101**, 93-105.

Conlon, F. L., Sedgwick, S. G., Weston, K. M. and Smith, J. C. (1996). Inhibition of Xbra transcription activation causes defects in mesodermal patterning and reveals autoregulation of Xbra in dorsal mesoderm. *Development* **122**, 2427-2435.

Cornell, R. A. and Kimelman, D. (1994). Activin-mediated mesoderm induction requires FGF. *Development* **120**, 453-62.

Cornell, R. A., Musci, T. J. and Kimelman, D. (1995). FGF is a prospective competence factor for early activin-type signals in Xenopus mesoderm induction. *Development* **121**, 2429-2437.

Cunliffe, V. and Smith, J. C. (1992). Ectopic mesoderm formation in Xenopus embryos caused by widespread expression of a Brachyury homologue. *Nature* **358**, 427-430.

Dale, L., Matthews, G. and Colman, A. (1993). Secretion and mesoderm-inducing activity of the TGF-beta-related domain of Xenopus Vg1. *EMBO J* **12**, 4471-4480.

Dohrmann, C. E., Kessler, D. S. and Melton, D. A. (1996). Induction of axial mesoderm by zDVR-1, the zebrafish orthologue of Xenopus Vg1. *Dev. Biol.* **175**, 108-17.

Dyson, S. and Gurdon, J. B. (1997). Activin signalling has a necessary function in Xenopus early development. *Curr. Biol.* **7**, 81-84.

Ecochard, V., Cayrol, C., Foulquier, F., Zaraisky, A. and Duprat, A. M. (1995). A novel TGF-beta-like gene, fugacin, specifically expressed in the Spemann organizer of Xenopus. *Dev. Biol.* **172**, 699-703.

Gammill, L. S. and Sive, H. L. (1997). Identification of otx2 target genes and restrictions in ectodermal competence during Xenopus cement gland formation. *Development* **124**, 471-481.

Gawantka, V., Delius, H., Hirschfeld, K., Blumenstock, C. and Niehrs, C. (1995). Antagonizing the Spemann organizer: role of the homeobox gene Xvent-1. *EMBO J* **14**, 6268-6279.

Graff, J. M., Thies, R. S., Song, J. J., Celeste, A. J. and Melton, D. A. (1994). Studies with a Xenopus BMP receptor suggest that ventral mesoderm-inducing signals override dorsal signals in vivo. *Cell* **79**, 169-179.

Glinka, A., Wu, W., Onichtouk, D., Blumenstock, C. and Niehrs, C. (1997). Head induction by simultaneous repression of BMP and Wnt signalling in Xenopus. *Nature* **389**, 517-519.

Green, J. B., Howes, G., Symes, K., Cooke, J. and Smith, J. C. (1990). The biological effects of XTC-MIF: quantitative comparison with Xenopus bFGF. *Development* **108**, 173-183.

Green, J. B., New, H. V. and Smith, J. C. (1992). Responses of embryonic Xenopus cells to activin and FGF are separated by multiple dose thresholds and correspond to distinct axes of the mesoderm. *Cell* **71**, 731-739.

Hansen, C. S., Marion, C. D., Steele, K., George, S. and Smith, W. C. (1997). Direct neural induction and selective inhibition of mesoderm and epidermis inducers by Xnr3. *Development* **124**, 483-92.

Harland, R. and Gerhart, J. (1997). Formation and function of Spemann's organizer. *Ann. Rev. Cell Dev. Biol.* **13**, 611-667.

Harland, R. M. (1991). In situ hybridization: an improved whole-mount method for Xenopus embryos. *Methods Cell Biol.* **36**, 685-695.

Harland, R. M. (1994). The transforming growth factor beta family and induction of the vertebrate mesoderm: bone morphogenetic proteins are ventral inducers [comment]. *Proc. Natl Acad. Sci. USA* **91**, 10243-10246.

Hawley, S. H., Wunnenberg-Stapleton, K., Hashimoto, C., Laurent, M. N., Watabe, T., Blumberg, B. W. and Cho, K. W. (1995). Disruption of BMP signals in embryonic Xenopus ectoderm leads to direct neural induction. *Genes Dev.* **9**, 2923-35.

Heasman, J. (1997). Patterning the Xenopus blastula. *Development* **124**, 4179-91.

Helde, K. A. and Grunwald, D. J. (1993). The DVR-1 (Vg1) transcript of zebrafish is maternally supplied and distributed throughout the embryo. *Dev. Biol.* **159**, 418-426.

Hemmati-Brivanlou, A., de la Torre, J. R., Holt, C. and Harland, R. M. (1991). Cephalic expression and molecular characterization of Xenopus En-2. *Development* **111**, 715-724.

Hemmati-Brivanlou, A. and Melton, D. A. (1992). A truncated activin receptor inhibits mesoderm induction and formation of axial structures in Xenopus embryos. *Nature* **359**, 609-614.

Hemmati-Brivanlou, A. and Melton, D. A. (1994). Inhibition of activin receptor signaling promotes neuralization in Xenopus. *Cell* **77**, 273-281.

Henry, G. L., Brivanlou, I. H., Kessler, D. S., Hemmati-Brivanlou, A. and Melton, D. A. (1996). TGF-beta signals and a pattern in Xenopus laevis endodermal development. *Development* **122**, 1007-1015.

Horb, M. E. and Thomsen, G. H. (1997). A vegetally localized T-box transcription factor in Xenopus eggs specifies mesoderm and endoderm and is essential for embryonic mesoderm formation. *Development* **124**, 1689-1698.

Hudson, C., Clements, D., Friday, R. V., Stott, D. and Woodland, H. R. (1997). Xsox17alpha and -beta mediate endoderm formation in Xenopus. *Cell* **91**, 397-405.

Isaacs, H. V., Pownall, M. E. and Slack, J. M. (1994). eFGF regulates Xbra expression during Xenopus gastrulation. *EMBO J* **13**, 4469-4481.

Isaacs, H. V., Tannahill, D. and Slack, J. M. (1992). Expression of a novel FGF in the Xenopus embryo. A new candidate inducing factor for mesoderm formation and anteroposterior specification. *Development* **114**, 711-720.

Jacobs, K. A., Collins-Racie, L. A., Colbert, M., Duckett, M., Golden-Fleet, M., Kelleher, K., Kriz, R., La Vallie, E. R., Merberg, D., Spaulding, V., Stover, J., Williamson, M. J. and McCoy, J. M. (1997). A genetic selection for isolating cDNAs encoding secreted proteins. *Gene* **198**, 289-296.

Jonas, E., Sargent, T. D. and Dawid, I. B. (1985). Epidermal keratin gene expressed in embryos of *Xenopus laevis*. *Proc. Nat. Acad. Sci., USA* **82**, 5413-5417.

Jones, C. M., Kuehn, M. R., Hogan, B. L., Smith, J. C. and Wright, C. V. (1995). Nodal-related signals induce axial mesoderm and dorsalize mesoderm during gastrulation. *Development* **121**, 3651-3662.

Jones, E. A. and Woodland, H. R. (1989). Spatial aspects of neural induction in Xenopus laevis. *Development* **107**, 785-791.

Joseph, E. M. and Melton, D. A. (1997). Xnr4: a Xenopus nodal-related gene expressed in the Spemann organizer. *Dev. Biol.* **184**, 367-372.

Joseph, E. M. and Melton, D. A. (1998). Mutant Vg1 ligands disrupt endoderm and mesoderm formation in Xenopus embryos. *Development* **125**, 2677-2685.

Kengaku, M. and Okamoto, H. (1995). bFGF as a possible morphogen for the anteroposterior axis of the central nervous system in Xenopus. *Development* **121**, 3121-3130.

Kessler, D. S. and Melton, D. A. (1995). Induction of dorsal mesoderm by soluble, mature Vg1 protein. *Development* **121**, 2155-2164.

Kimelman, D. and Griffin, K. J. (1998). Mesoderm induction: a postmodern view [comment]. *Cell* **94**, 419-421.

Kingsley, D. M. (1994). The TGF-beta superfamily: new members, new receptors, and new genetic tests of function in different organisms. *Genes Dev.* **8**, 133-146.

Kintner, C. R. and Melton, D. A. (1987). Expression of Xenopus N-CAM RNA in ectoderm is an early response to neural induction. *Development* **99**, 311-325.

Kolm, P. J., Apekin, V. and Sive, H. L. (1997). Xenopus hindbrain patterning requires retinoid signaling. *Dev. Biol.* **192**, 1-16.

Kolm, P. J. and Sive, H. L. (1995). Regulation of the Xenopus labial homeodomain genes, HoxA1 and HoxD1: activation by retinoids and peptide growth factors. *Dev. Biol.* **167**, 34-49.

Kolm, P. J. and Sive, H. L. (1997). In *Cold Spring Harbor Symposia on Quantitative Biology* **LXII.**), pp. 511-521. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Krieg, P. A. and Melton, D. A. (1984). Functional messenger RNAs are produced by SP6 in vitro transcription of cloned cDNAs. *Nucleic Acids Res.* **12**, 7057-7070.

Kroll, K. L. and Amaya, E. (1996). Transgenic Xenopus embryos from sperm nuclear transplantations reveal FGF signaling requirements during gastrulation. *Development* **122**, 3173-3183.

La Bonne, C. and Whitman, M. (1994). Mesoderm induction by activin requires FGF-mediated intracellular signals. *Development* **120**, 463-472.

La Vallie, E. R., Di Blasio, E. A., Kovacic, S., Grant, K. L., Schendel, P. F. and McCoy, J. M. (1993). A thioredoxin gene fusion expression system that circumvents inclusion body formation in the E. coli cytoplasm. *Biotechnology (NY)* **11**, 187-193.

Lamb, T. and Harland, R. (1995). Fibroblast growth factor is a direct neural inducer, which combined with noggin generates anterior-posterior neural pattern. *Development* **121**, 3627-3636.

Lemaire, P., Garrett, N. and Gurdon, J. B. (1995). Expression cloning of Siamois, a Xenopus homeobox gene expressed in dorsal-vegetal cells of blastulae and able to induce a complete secondary axis. *Cell* **81**, 85-94.

Lu, Z., Di Blasio-Smith, E. A., Grant, K. L., Warne, N. W., La Vallie, E. R., Collins-Racie, L. A., Follettie, M. T., Williamson, M. J. and McCoy, J. M. (1996). Histidine patch thioredoxins. Mutant forms of thioredoxin with metal chelating affinity that provide for convenient purifications of thioredoxin fusion proteins. *J. Biol. Chem.* **271**, 5059-5065.

Lustig, K. D., Kroll, K., Sun, E., Ramos, R., Elmendorf, H. and Kirschner, M. W. (1996a). A Xenopus nodal-related gene that acts in synergy with noggin to induce complete secondary axis and notochord formation. *Development* **122**, 3275-3282.

Lustig, K. D., Kroll, K. L., Sun, E. E. and Kirschner, M. W. (1996b). Expression cloning of a Xenopus T-related gene (Xombi) involved in mesodermal patterning and blastopore lip formation. *Development* **122**, 4001-4012.

McGrew, L. L., Hoppler, S. and Moon, R. T. (1997). Wnt and FGF pathways cooperatively pattern anteroposterior neural ectoderm in Xenopus. *Mech. Dev.* **69**, 105-114.

Mohun, T. J., Brennan, S., Dathan, N., Fairman, S. and Gurdon, J. B. (1984). Cell type-specific activation of actin genes in the early amphibian embryo. *Nature* **311**, 716-721.

Nieuwkoop, P. D. and Faber, J. (1994). *Normal Tables of Xenopus laevis* (Daudin). New York and London: Garland Publishing, Inc.

Northrop, J. L. and Kimelman, D. (1994). Dorsal-ventral differences in Xcad-3 expression in response to FGF-mediated induction in Xenopus. *Dev. Biol.* **161**, 490-503.

Pannese, M., Polo, C., Andreazzoli, M., Vignali, R., Kablar, B., Barsacchi, G. and Boncinelli, E. (1995). The Xenopus homologue of Otx2 is a maternal homeobox gene that demarcates and specifies anterior body regions. *Development* **121**, 707-720.

Rehemtulla, A. and Kaufman, R. J. (1992). Protein processing within the secretory pathway. *Curr. Opin. Biotechnol.* **3**, 560-565.

Ruiz i Altaba, A. and Jessell, T. M. (1992). Pintallavis, a gene expressed in the organizer and midline cells of frog embryos: involvement in the development of the neural axis. *Development* **116**, 81-93.

Ryan, K., Garrett, N., Mitchell, A. and Gurdon, J. B. (1996). Eomesodermin, a key early gene in Xenopus mesoderm differentiation. *Cell* **87**, 989-1000.

Saha, M. S. and Grainger, R. M. (1992). A labile period in the determination of the anterior-posterior axis during early neural development in Xenopus. *Neuron* **8**, 1003-1014.

Sasai, Y., Lu, B., Piccolo, S. and De Robertis, E. M. (1996). Endoderm induction by the organizer-secreted factors chordin and noggin in Xenopus animal caps. *EMBO J* **15**, 4547-4555.

Schlunegger, M. P., Cerletti, N., Cox, D. A., McMaster, G. K., Schmitz, A. and Grutter, M. G. (1992). Crystallization and preliminary X-ray analysis of recombinant human transforming growth factor beta 2. *FEBS Lett.* **303**, 91-93.

Schulte-Merker, S. and Smith, J. C. (1995). Mesoderm formation in response to Brachyury requires FGF signalling. *Curr. Biol.* **5**, 62-67.

Seleiro, E. A., Connolly, D. J. and Cooke, J. (1996). Early developmental expression and experimental axis determination by the chicken Vg1 gene. *Curr. Biol.* **6**, 1476-1486.

Shah, S. B., Skromne, I., Hume, C. R., Kessler, D. S., Lee, K. J., Stern, C. D. and Dodd, J. (1997). Misexpression of chick Vg1 in the marginal zone induces primitive streak formation. *Development* **124**, 5127-5138.

Sharpe, C. R., Fritz, A., De Robertis, E. M. and Gurdon, J. B. (1987). A homeobox-containing marker of posterior neural differentiation shows the importance of predetermination in neural induction. *Cell* **50**, 749-758.

Sive, H. L. and Cheng, P. F. (1991). Retinoic acid perturbs the expression of *Xhox.lab* genes and alters mesodermal determination in *Xenopus laevis*. *GenesDev.* **5**, 1321-1332.

Sive, H. L., Hattori, K. and Weintraub, H. (1989). Progressive determination during formation of the anteroposterior axis in Xenopus laevis. *Cell* **58**, 171-180.

Smith, J. C., Price, B. M., Green, J. B., Weigel, D. and Herrmann, B. G. (1991). Expression of a Xenopus homolog of Brachyury (T) is an immediate-early response to mesoderm induction. *Cell* **67**, 79-87.

Smith, W. C., McKendry, R., Ribisi, S., Jr. and Harland, R. M. (1995). A nodal-related gene defines a physical and functional domain within the Spemann organizer. *Cell* **82**, 37-46.

Sokol, S., Christian, J. L., Moon, R. T. and Melton, D. A. (1991). Injected Wnt RNA induces a complete body axis in Xenopus embryos. *Cell* **67**, 741-752.

Song, J. and Slack, J. M. (1996). XFGF-9: a new fibroblast growth factor from Xenopus embryos. *Dev. Dynam.* **206**, 427-436.

Stennard, F., Carnac, G. and Gurdon, J. B. (1996). The Xenopus T-box gene, Antipodean, encodes a vegetally localised maternal mRNA and can trigger mesoderm formation. *Development* **122**, 4179-4188.

Suzuki, A., Thies, R. S., Yamaji, N., Song, J. J., Wozney, J. M., Murakami, K. and Ueno, N. (1994). A truncated bone morphogenetic protein receptor affects dorsal-ventral patterning in the early Xenopus embryo. *Proc. Natl Acad. Sci. USA* **91**, 10255-10259.

Taira, M., Jamrich, M., Good, P. J. and Dawid, I. B. (1992). The LIM domain-containing homeo box gene Xlim-1 is expressed specifically in the organizer region of Xenopus gastrula embryos. *Genes Dev.* **6**, 356-366.

Thomsen, G., Woolf, T., Whitman, M., Sokol, S., Vaughan, J., Vale, W. and Melton, D. A. (1990). Activins are expressed early in Xenopus embryogenesis and can induce axial mesoderm and anterior structures. *Cell* **63**, 485-493.

Thomsen, G. H. and Melton, D. A. (1993). Processed Vg1 protein is an axial mesoderm inducer in Xenopus. *Cell* **74**, 433-441.

von Dassow, G., Schmidt, J. E. and Kimelman, D. (1993). Induction of the Xenopus organizer: expression and regulation of Xnot, a novel FGF and activin-regulated homeo box gene. *Genes Dev.* **7**, 355-366.

von Heijne, G. (1986). A new method for predicting signal sequence cleavage sites. *Nucleic Acids Res.* **14**, 4683-4690.

Weeks, D. L. and Melton, D. A. (1987). A maternal mRNA localized to the vegetal hemisphere in Xenopus eggs codes for a growth factor related to TGF-beta. *Cell* **51**, 861-867.

Zhang, J., Houston, D. W., King, M. L., Payne, C., Wylie, C. and Heasman, J. (1998). The role of maternal VegT in establishing the primary germ layers in *Xenopus* embryos. *Cell* **94**, 515-524.

Zhang, J. and King, M. L. (1996). Xenopus VegT RNA is localized to the vegetal cortex during oogenesis and encodes a novel T-box transcription factor involved in mesodermal patterning. *Development* **122**, 4119-4129.

Zoltewicz, J. S. and Gerhart, J. C. (1997). The Spemann organizer of Xenopus is patterned along its anteroposterior axis at the earliest gastrula stage. *Dev. Biol.* **192**, 482-491.