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

Benjamin I. Sun 1, Sara M. Bush 1, Lisa A. Collins-Racie 2, Edward R. LaVallie 2, Elizabeth A. DiBlasio-Smith 2, Neil M. Wolfman 2, John M. McCoy 2 and Hazel L. Sive 1,*

1 Whitehead Institute for Biomedical Research and Massachusetts Institute of Technology, Nine Cambridge Center, Cambridge MA 02142, USA
2 Genetics Institute, 87 CambridgePark Drive, Cambridge MA 02140, USA

*Author for correspondence (e-mail: sive@wi.mit.edu)

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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 Xnr3, activin and an activated form of Vg1 are also able to

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induce, in normal embryos, a secondary axis lacking a head (Thomensen 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 (Komi, 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 XhoI site (5′-AACTGCTGGTGATCATCGGAGNNNNN-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 EcoRI adapter: BIS1, 5′-AATTCCGGACTACTACAGGGTGC-3′, unphosphorylated and BIS2, 5′-CACCT-GTAGAGTCCG-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 EcoRI end at the 5′ end of cDNA before XhoI digestion at the 3′ end. The cDNA was directionally cloned into the SST vector (pSUC277M13ORI, 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 XhoI site, 5′-CTCGAGTITTGGTTTTTTTTTTTTTTTTT-3′, was used along with methylated dCTP for first-strand synthesis; (2) two oligos were used for EcoRI adapter: 5′-AATTCCGATCCCAACAAACAGTA-3′ and 5′-TACT-GTTTGTTGTATGGG-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 XhoI digestion. This cDNA was cloned directionally into EcoRI-XhoI double-digested CS2+ vector. The resulting library contains 5×10⁶ 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 was isolated by standard hybridization with probe synthesized using oligo 5′-GGAGGTATAGCCAACACTCTGTCCATG-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 EcoRII 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: EcoRI linearized pSP64TBVg1 (for BVg1; Thomsen and Melton, 1993), pPB6-4T (for activin; Sokol et al., 1991), pXFD/Xss (for XFD, Amaya et al., 1991); NotI linearized Xnr1/CS2+ (for Xnr1; Lustig et al., 1996a), pCS2+/VegTc (for VegT, Zhang and King, 1996), pAmi-2 (for Xnr3, Smith et al., 1995), derrière/CS2+ and Cm-derrière/CS2+; Smal linearized Xbra/sp64T (for Xbra, Smith et al., 1991); Xbal linearized Xnr4-64TNE (for Xnr4, Joseph and Melton, 1997); KpnI 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 digoxigenin-11-UTP (Harland, 1991). Knox20, en-2, otz2, HoxB9 and XCG were as described (Kolm et al., 1997). Other templates were as follows: derrière/CS2+, EcoRII linearized, T7 transcribed; pBSC100 (for mactin), EcoRII linearized, T3 transcribed; pSP70-N1 (for NCAM; Kintner and Melton, 1987), EcoRV 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 sequence of other primers used are as follows (sense primer first): ODC (21 cycles): 5'-CAACGTGTAGGGCTCTGAT-3' and 5'-CATATAAAAGGGTTGTCTCTGTA-3', Xbra (24 cycles): 5'-TCTTTAGGATGAGTCTGC-3' and 5'-GTTGATCTGGTCCCAG-3', TgaT (28 cycles): 5'-TTAGCTTCCAGAGCAGT-3' and 5'-CCACATAGCCTGGGGAATC-3', eFGF (27 cycles): 5'-CGGTTTCTATATCAGTGT-3' and 5'-GCGTTATAGTGTGTGCAAG-3', gsc (26 cycles): 5'-GATTATAAACAGCAGCTTGG-3' and 5'-TGTAAAGGGAGCA-TGCCTGAGG-3', eomes (25 cycles): 5'-GGGCAAACAGCACAGAATTAC-3' and 5'-TGGAGGGCGACATAAGGGAGAT-3', Xlim-1 (25 cycles): 5'-GTGATGCTGCTTCATCTCTTCA-3' and 5'-GACACAGCCGCACATTTGTA-3', Pintallavis (25 cycles): 5'-GACGGCACCACCACAGATG-3' and 5'-CCAGATTTGGG-3'

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 localization 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-β molecules (xVg1, 76% (zVg1, Helde and Grunwald, 1993) and 73% (cVg1, Seleiro et al., 1996).

The expression pattern of derrière suggests a role in posterior determination
In order to ask what role derrière might play during

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'-GCAATGGTGCAGGGAGTACT-3' and 5'-CATCATGACCTTGGGGAATC-3', sense, paired with T7 primer) and 5'-GCATGGTGCAGGGAGTACT-3' (antisense, paired with SP6 primer) using derrière/CS2+ as template. The resulting 5' (EcoRI-Sall double-digested) and 3' (SalI-XhoI double-digested) ends of Cm-derrière were subcloned into CS2+ (EcoRI-Sall double-digested) by a three-piece ligation and the final construct was confirmed by sequence determination.
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 lateral side of the intercellular spaces.
derrière regulates posterior development

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 stage 11.5 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 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.

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.
(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

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**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, Pintallavias 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 thomomerses 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 doses 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 Vg1 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, it 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
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 derrière by wild-type derrière and VegT. 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 Veg1. 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
Other members of this family have been found in chick (cVgI) and zebrafish (zVgI). All Vg family members share the ability to induce mesoderm and a secondary axis (Seleiro et al., 1996; Shah et al., 1997). Interestingly, cVgI, like derrière, is expressed posteriorly, suggesting that the function of derrière and cVgI may be similar. In contrast, zVgI expression is maternal and ubiquitous, although, since the protein persists through gastrulation, zVgI may have a later function (Helde and Grunwald, 1993; Doehrmann et al., 1996).

The expression of Xenopus VgI RNA suggests a role for this gene in mesoderm and endoderm induction, however, the VgI proprotein is inefficiently processed and native VgI RNA displays no activity when overexpressed (Dale et al., 1993). The mesoderm-inducing and axis-duplication activity of derrière is similar to that of BVgI (Dale et al., 1993; Thomsen and Melton, 1993), raising the possibility that BVgI may partially phenocopy the effects of ectopic derrière. However, several results suggest that derrière and VgI have different activities. First, unlike derrière, BVgI does not suppress head formation (Dale et al., 1993) and, second, lineage tracer co-injected with BVgI is found exclusively in the endoderm, consistent with the observation that high concentrations of BVgI 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 VgI (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 neurodermal 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 neurodermal 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-EnR, 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, FG9 (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-β 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
Vg, vegetal pole; A, anterior; P, posterior; meso, mesodermal fates; endo, endodermal fates.

excluded from anterior mesoderm and from the dorsal midline (close hatching), and continues to promote posterolateral fates. An, animal pole; play a role in activating posterolateral (paraxial) mesodermal fates at this time, before somite formation. By mid to late gastrula, expression is maintained in the mesoderm by a positive feedback loop that includes early gastrula stages, (not shown) or a dominant negative -activin activity. At the mid-blastula transition, we speculate that maternal transcription factors (such as Veg T, zygotically expressed) establish between mid-blastula transition. Soon after this, a regulatory loop is involved with 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 that 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 Veg T activates expression of derrière soon after the mid-blastula transition. Soon after this, a regulatory loop is established between derrière, zygotically expressed Veg T, 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 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 Veg T, 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, Veg T and derrière suggested by these studies, and addressing further the mechanism by which derrière regulates posterior patterning.

Fig. 10. Model for derrière activity. At the mid-blastula transition, we speculate that maternal transcription factors (such as Veg T) 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 feedback loop that includes FGFs (such as eFGF), Xbra and zygotic Veg T (close hatching). derrière expression in the endoderm is not maintained presumably because such a feedback 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.

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