

## ***derrière*: a TGF- $\beta$ family member required for posterior development in *Xenopus***

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### SUMMARY

TGF- $\beta$  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- $\beta$  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 TGF $\beta$  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- $\beta$ , *derrière*, *Vg1*, *VegT*, Mesoderm, Posterior, Dominant negative

### INTRODUCTION

Members of the TGF $\beta$  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- $\beta$  receptors in whole embryos (Hemmati-Brivanlou and Melton, 1992). Maternally expressed TGF- $\beta$ s 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  $\beta$ -catenin (Heasman, 1997), TGF- $\beta$  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- $\beta$  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- $\beta$  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- $\beta$ s 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- $\beta$  signaling. Despite these intriguing data, however, a normal role for TGF- $\beta$  family members in posterior patterning has not been defined, since no TGF- $\beta$  with localized expression or endogenous activity consistent with such a role has been described.

We have begun to address the role TGF- $\beta$  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- $\beta$  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- $\beta$  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  $\mu$ g) 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  $\mu$ g 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'-AAGCTTGGCGGTCTCGAGNNNNNNNNN-3') was used at 60  $\mu$ M 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'-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 *EcoRI* end at the 5' end of cDNA before *XhoI* 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 *XhoI* site, 5'-CTCGAGTTTTTTTTTTTTT-3', was used along with methylated dCTP for first-strand synthesis; (2) two oligos were used for *EcoRI* adapter: 5'-AATTCCCATAGCAACAAACAGTA-3' and 5'-TACT-GTTTGTGCTATGGG-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 $\times$ 10<sup>6</sup> 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'-GAAAGTGATAGCCACAACCTGCCATG-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 *EcoRI* using the primer 5'-CCCTTAGCATTTCGTCAGT-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).  $\beta$ -globin and *lacZ* were as described (Kolm et al., 1997). Other templates were as follows: *EcoRI* linearized pSP64TBVg1 (for *BVg1*; Thomsen and Melton, 1993), p $\beta$ B-64T (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+VegTfc (for *VegT*, Zhang and King, 1996), pdor3 (for *Xnr3*, Smith et al., 1995), *derrière*/CS2+ and *Cm-derrière*/CS2+; *SmaI* linearized *Xbra*/pSP64T (for *Xbra*, Smith et al., 1991); *XbaI* 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). *Krox20*, *en-2*, *otx2*, *HoxB9* and *XCG* were as described (Kolm et al., 1997). Other templates were as follows: *derrière*/CS2+, *EcoRI* linearized, T7 transcribed; pBSAC100 (for *m-actin*), *EcoRI* 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,  $\beta$ -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.

### $\beta$ -galactosidase staining and in situ hybridization

$\beta$ -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-Briavanlou 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'-TTAGCTCCAG-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'-GGGCAACAGCAC-AAGAATAC-3' and 5'-TGGAGGCGCATAAGGGAAGAT-3', *Xlim-1* (25 cycles): 5'-GTGTCTGCCTTCTATTCTCCTAA-3' and 5'-GCACAGCCCGCACACTTGGTA-3', *Pintallavis* (25 cycles): 5'-GCAGGCACCAACAAGATGAT-3' and 5'-CCAGATTCGGG-GTGCAGAGT-3', *Xnot* (26 cycles): 5'-CAGACCTGCCTCCAAA-CTATCC-3' and 5'-TCTCCCCTGGGCATCCTCATT-3', *siamois* (27 cycles): 5'-AGGAACCCACAGGATAAAT-3' and 5'-GTTG-ACTGCAGACTGTTGACTA-3', *Xvent-1* (25 cycles): 5'-GCATC-TCCTTGGCATAATTTGG-3' and 5'-TTCCCTTCAGCATGGTTCAA-C-3', *XK81* (19 cycles): 5'-TCATTCGGTTCAGCTCTTCTTAC-3' and 5'-TCCAGGGCTTACTTTCTCCAG-3', *Xsox17 $\alpha$*  (25 cycles): 5'-CAATGGCAGCTACCCTACC-3' and 5'-CTTGGCCACATAG-CTCAGATAC-3', *endodermin* (21 cycles): 5'-TATTCTGACT-CCTGAAGGTG-3' and 5'-GAGAAGTGCCTATGTGCCTC-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 *NdeI* and *XbaI* restriction sites at the 5'/3' ends of *derrière*. This allowed cloning of the ligated duplexes into *NdeI-XbaI*-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<sub>4</sub> and 100  $\mu$ g/ml ampicillin) to an OD<sub>550</sub> of 0.05. The culture was grown at 30°C until the OD<sub>550</sub> reached 0.5, then L-tryptophan was added to a concentration of 100  $\mu$ g/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 $\times$  MBS; bFGF (Promega) was used at 100 ng/ml (plus 200 ng/ml BSA) in 0.5 $\times$  MBS; *Derrière* was used at 200 ng/ml (plus 100 ng/ml BSA) in 0.5 $\times$  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'-GCATGGTTCGACGGGAGTACT-CATTCATCACCTC-3' (sense, paired with T7 primer) and 5'-GC-ATGGTTCGACGCCTTGAGTTTTGCAATTGGATG-3' (antisense, paired with SP6 primer) using *derrière*/CS2+ as template. The resulting 5' (*EcoRI-SalI* double-digested) and 3' (*Sall-XhoI* double-digested) ends of Cm-*derrière* were subcloned into CS2+ (*EcoRI-XhoI* double-digested) by a three-piece ligation and the final construct was confirmed by sequence determination.

## RESULTS

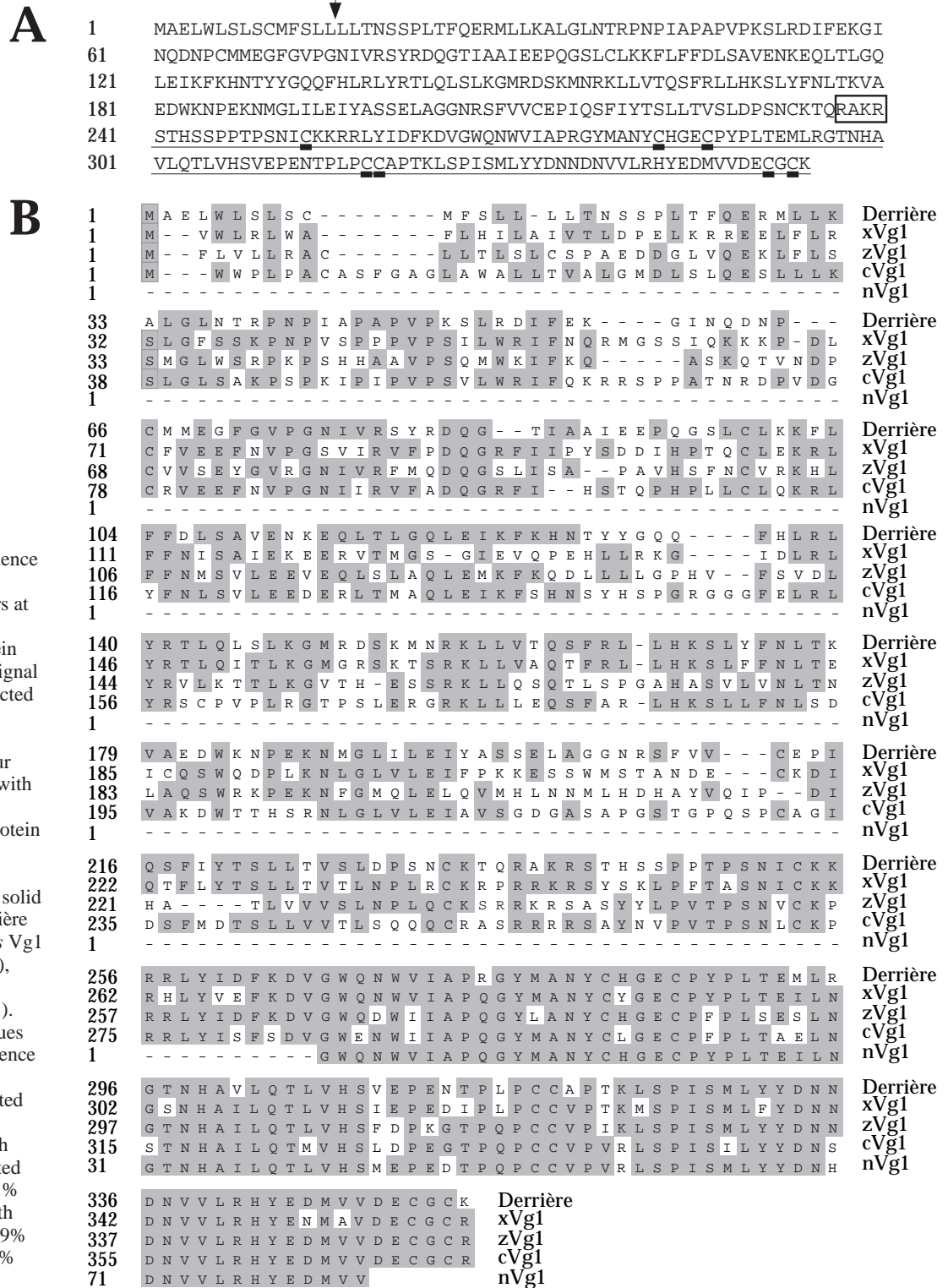
### *derrière* is a new member of the TGF- $\beta$ superfamily

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- $\beta$  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- $\beta$  molecules (Kingsley, 1994). *derrière* is a member of the *Vg*-related family of TGF- $\beta$ s 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- $\beta$ s 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

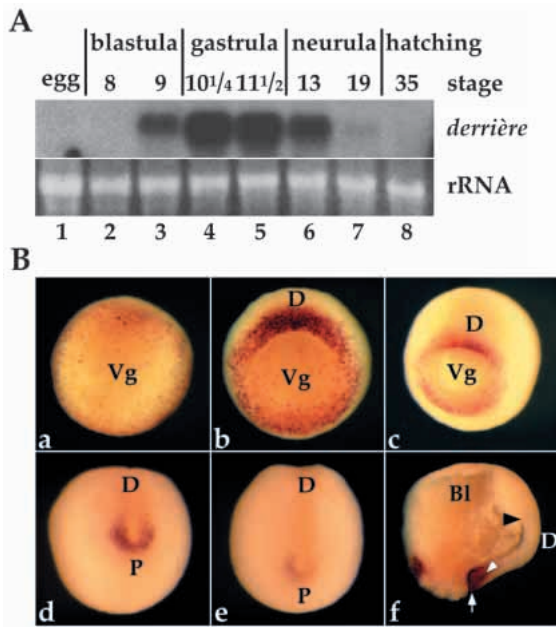
In order to ask what role *derrière* might play during



**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



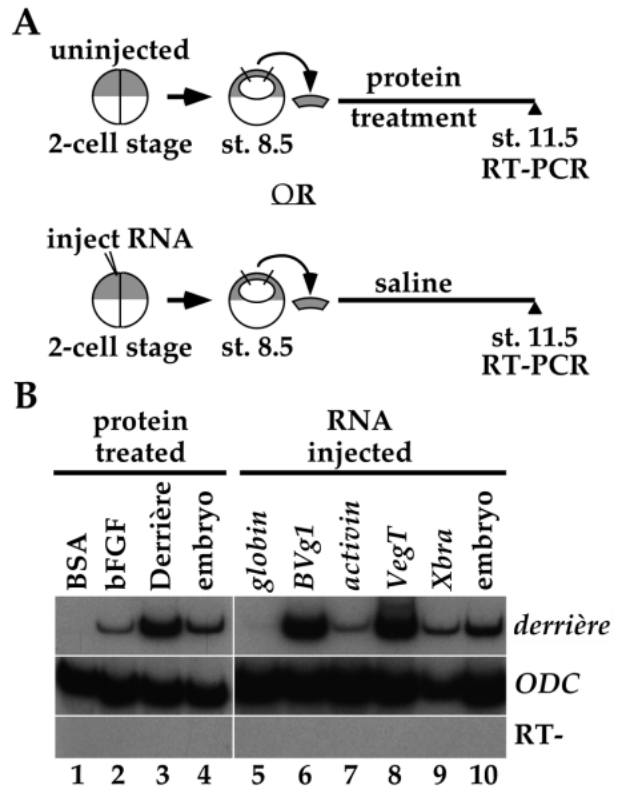
**Fig. 2.** Temporal and spatial expression patterns of *derrière*. (A) Northern analysis of *Xenopus* embryos. One embryo equivalent per lane was analyzed for *derrière* RNA (top row) at various embryonic stages shown. Ethidium-bromide-stained 28S rRNA is a loading control (bottom row). Lanes as marked. (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.

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,



**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.

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- $\beta$  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

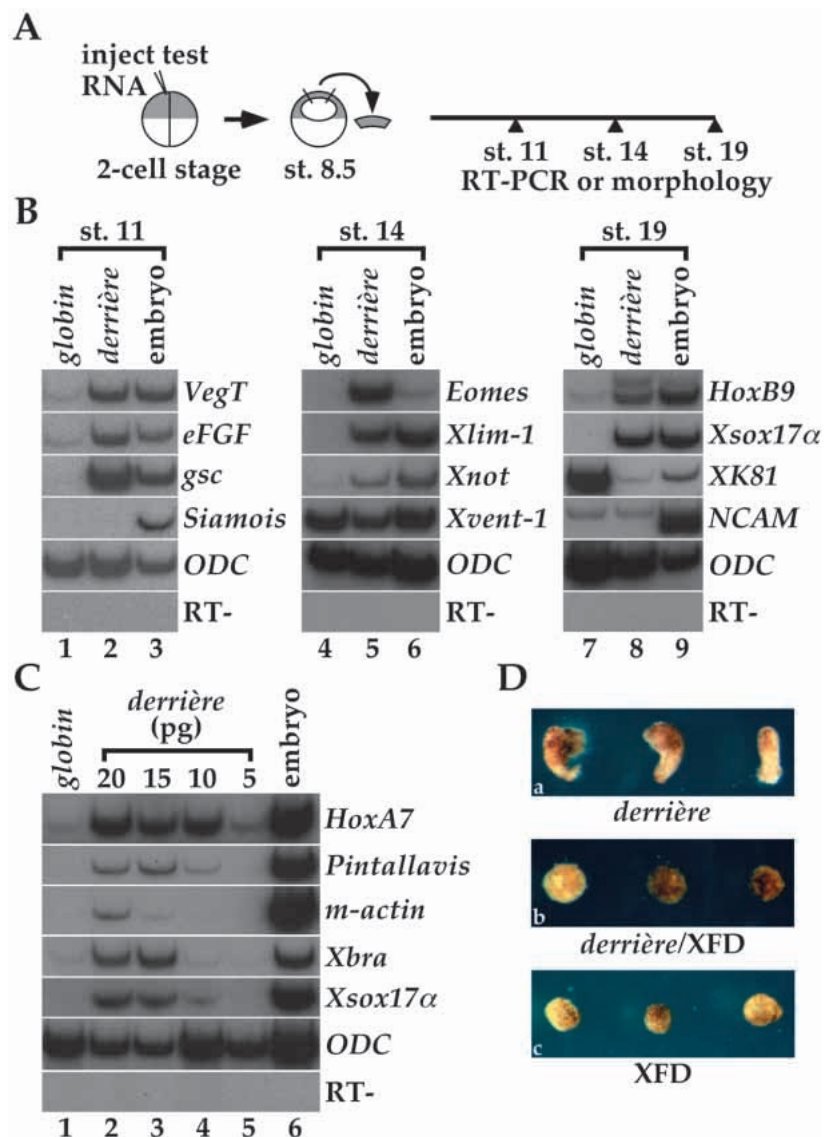
*derrière*, the posterior marker *eFGF* (Isaacs et al., 1992), the prechordal plate marker *gsc* (Cho et al., 1991), the mesendodermal marker *eomesodermin* (Ryan et al., 1996), the dorsal mesodermal markers *Xlim-1* (Taira et al., 1992) and *Xnot* (von Dassow et al., 1993). Additionally, *derrière* induced expression of the posterior mesodermal and ectodermal markers *HoxB9* (Sharpe et al., 1987) and an endodermal marker, *Xsox17 $\alpha$*  (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.

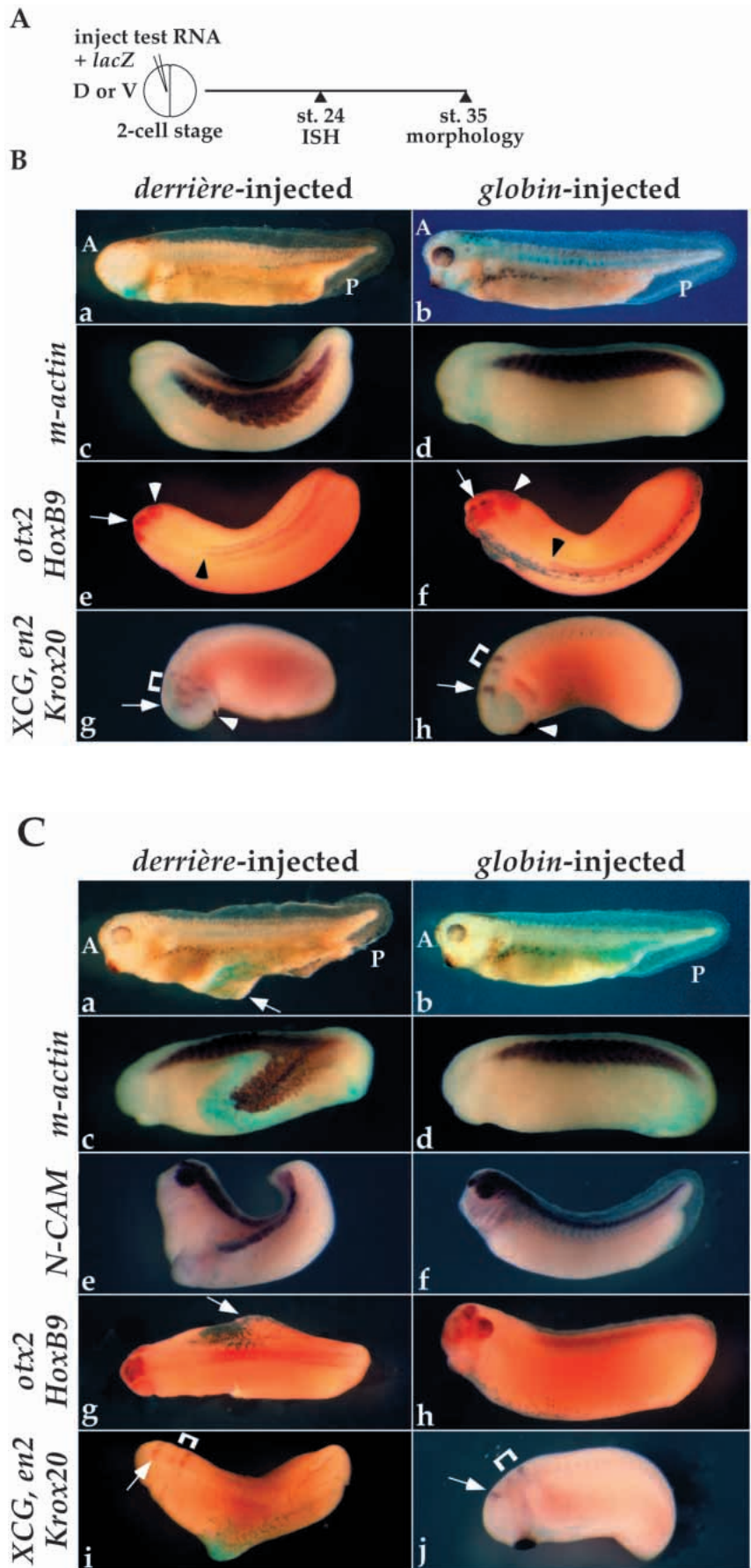
Animal caps were removed from stage 8.5 embryos and were cultured until sibling embryos reached stages indicated by the triangles. Animal caps and whole embryos were harvest for RT-PCR or morphology. (B) Expression of marker genes (see Results) in animal caps after injection of 400 pg RNA. Injection 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. *Xsox17 $\alpha$*  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.  $\beta$ -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- $\beta$ s, 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 *Xsox17 $\alpha$*  were induced (lane 2), at 15 pg *HoxA7*, *Pintallavis*, *Xbra* and *Xsox17 $\alpha$*  were induced (lane 3) while, at 10 pg, only *HoxA7* and low levels of *Pintallavis* and *Xsox17 $\alpha$*  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  $\beta$ -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 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 *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- $\beta$  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 mesoderm (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).

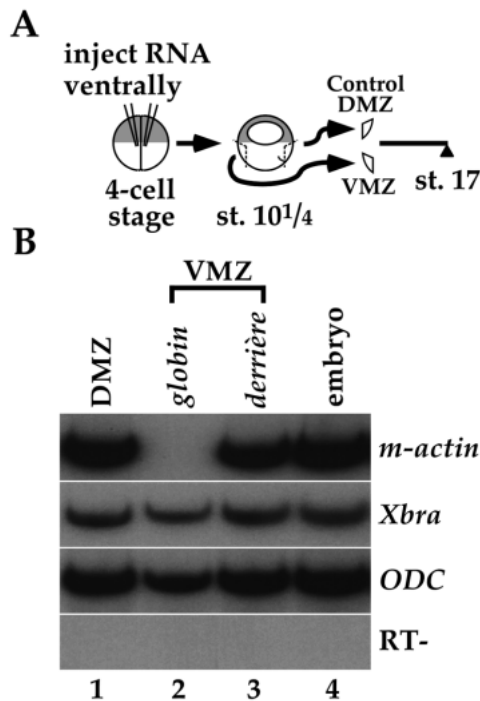
At the time of dissection, misexpressed *derrière* induced a secondary invagination on the ventral side of the embryo (not shown) similar to that observed after *VegT* misexpression (Lustig et al., 1996b). At the time of harvest, *globin*-injected VMZ explants remained round (0% elongation;  $n=30$ ) 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

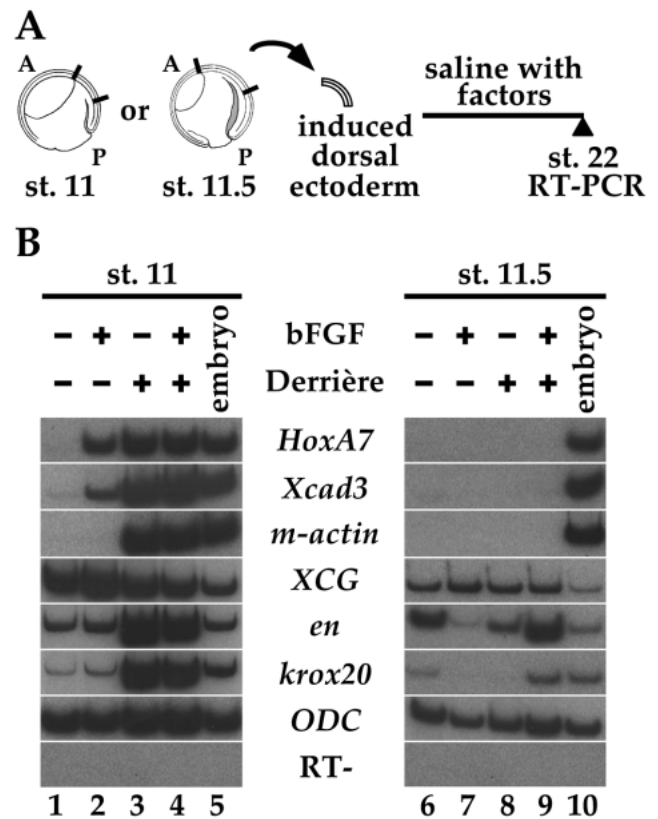




**Fig. 6.** *derrière* increases dorsal character of the ventral marginal zone. (A) Experimental scheme. 25 pg *derrière* RNA was injected into the marginal zone of both ventral blastomeres of 4-cell stage wild-type embryos. The VMZ was dissected at stage 10.25 and cultured until harvest for RT-PCR at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

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*

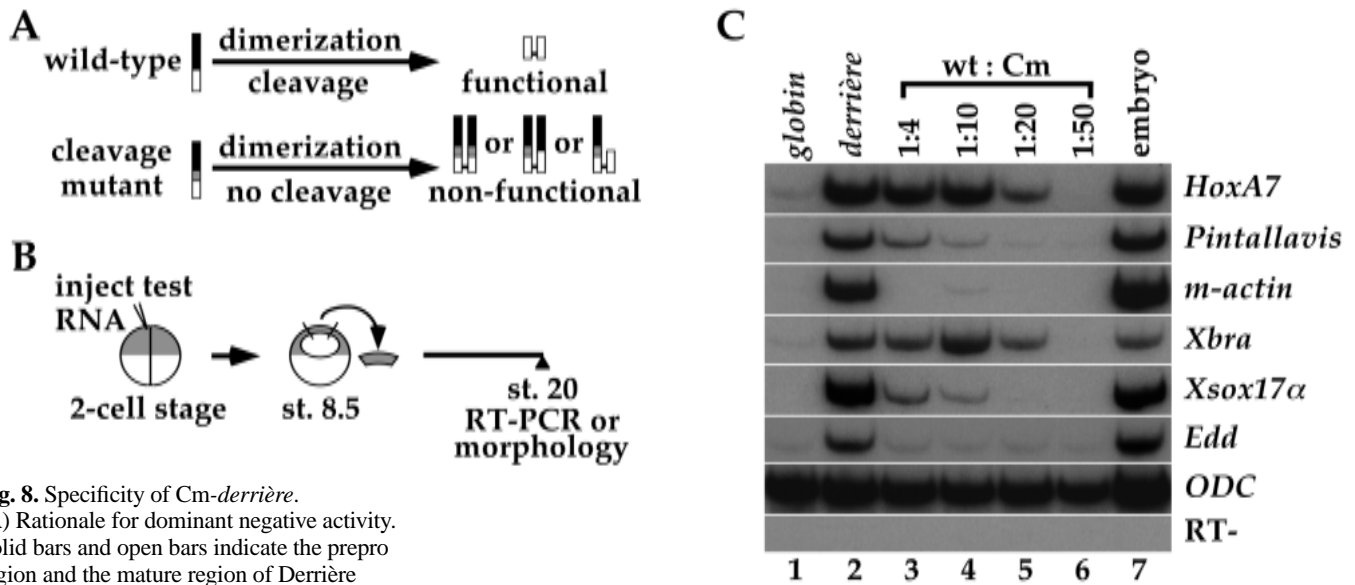


**Fig. 7.** *Derrière* posteriorizes isolated neurectoderm indirectly. (A) Experimental scheme. Anterior dorsal ectoderm (aDE, indicated by the cut marks, see Materials and Methods) was isolated from stage 11 and 11.5 (mid-gastrula) embryos. Explants were cultured in saline alone or with added factors 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.

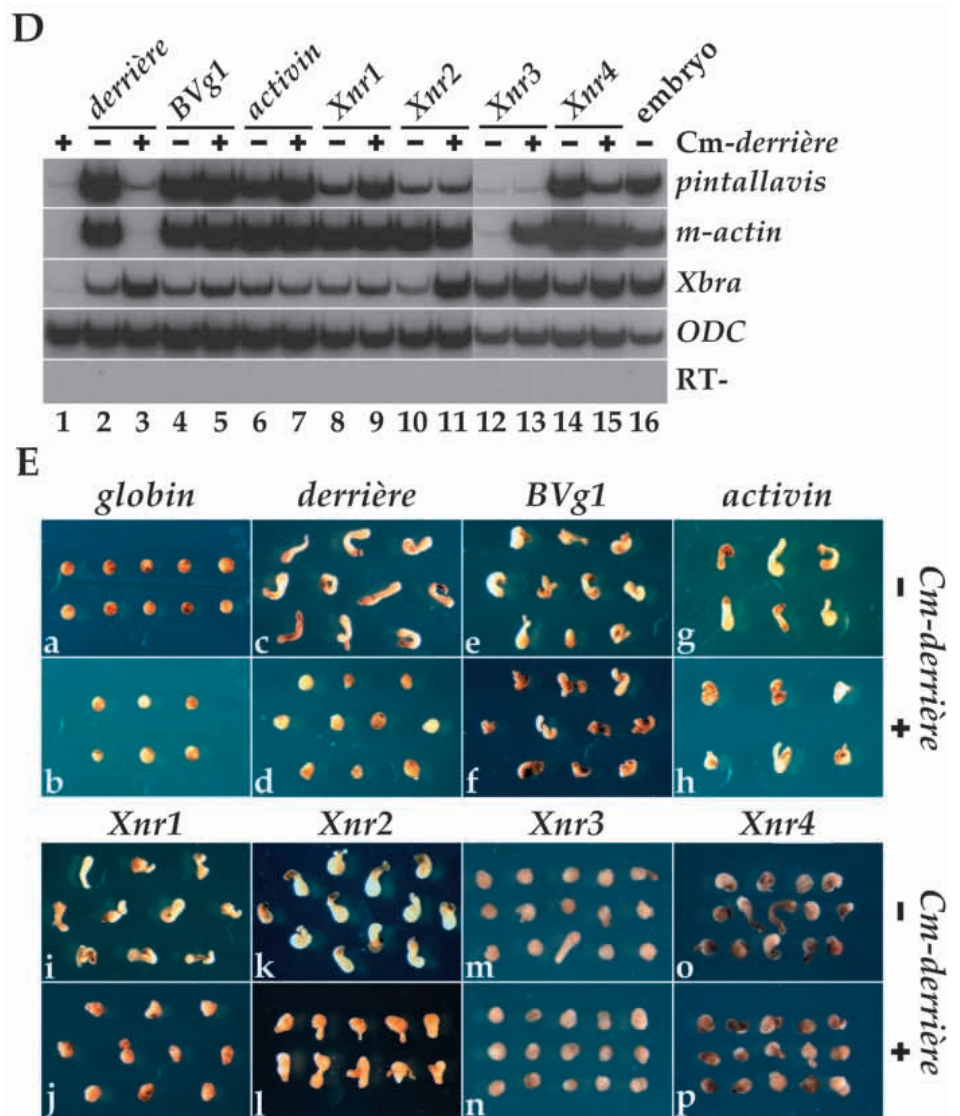
could induce mesoderm and concomitantly induce expression of posterior neural markers in anterior neurectoderm.

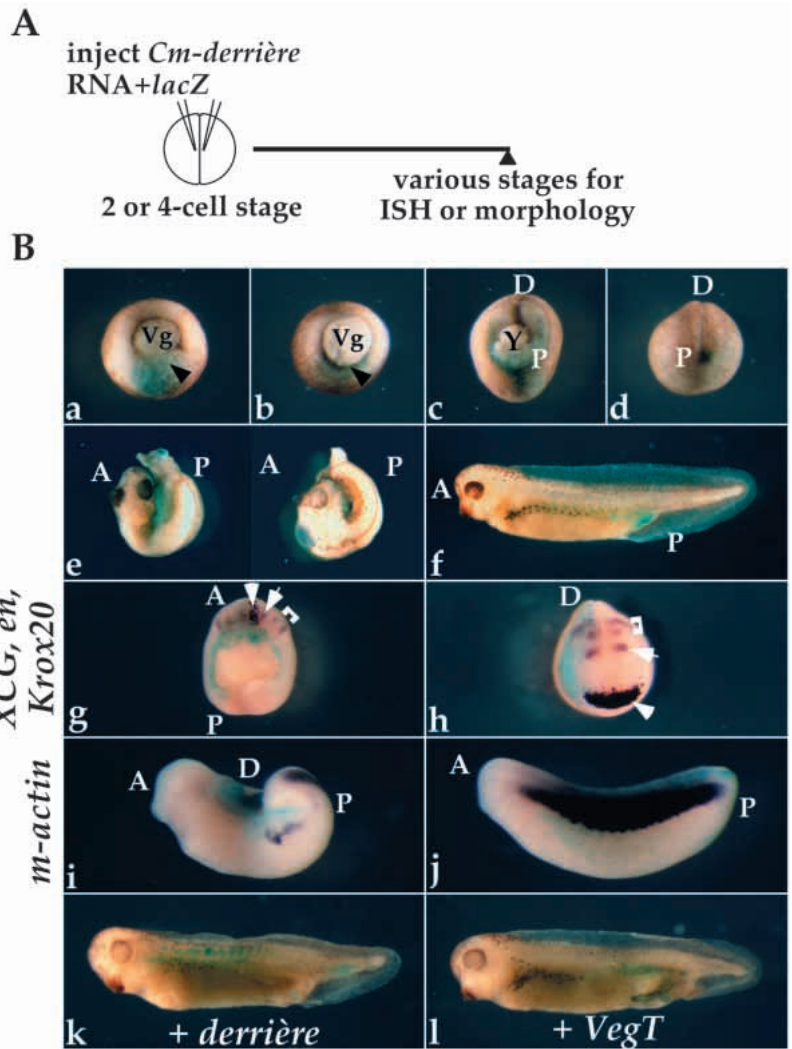
Ectodermal explants of a slightly later stage (stage 11.5) expressed *XCG*, *en-2* and *krox20* after culture, but failed to express *HoxA7*, *Xcad3* or *m-actin* (lane 6). bFGF did not induce any posterior markers, had no effect on *XCG* expression and slightly decreased both *en-2* and *krox20* expression (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- $\beta$  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- $\beta$  family members: animal cap elongation assay. *Cm-derrière* was used at a 10:1 ratio to co-injected TGF- $\beta$ s, 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  $\beta$ -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* protein to the mature form. Since TGF- $\beta$ s 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*, *Xsox17 $\alpha$*  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 *Xsox17 $\alpha$*  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- $\beta$  family members (Fig. 8B). RNAs encoding various TGF- $\beta$  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- $\beta$  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- $\beta$  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- $\beta$  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- $\beta$  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- $\beta$  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.

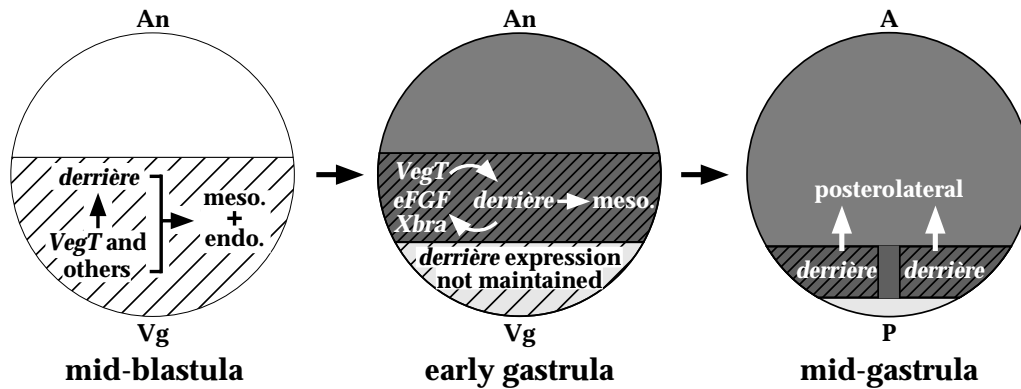
#### ***derrière* may be induced as part of a regulatory loop with *VegT* and *FGF***

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<sup>R</sup>*, 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- $\beta$  signaling is required for normal endoderm and mesoderm formation (Kimelman and Griffin, 1998). *derrière* is a good candidate to be an endogenous TGF- $\beta$  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- $\beta$ s 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 *derriere* activity. At the mid-blastula transition, we speculate that maternal transcription factors (such as *VegT*) and possibly secreted factors activate *derriere* expression in the presumptive mesoderm and endoderm (wide hatching). During early gastrula, *derriere* expression is maintained in the mesoderm by a positive feed-back loop that includes *FGFs* (such as *eFGF*, *Xbra* and zygotic *VegT* (close hatching)). *derriere* expression in the endoderm is not maintained presumably because such a feed-back loop cannot be established there. During early gastrula stages, *derriere* may activate mesendodermal fates in both the future head region and more posteriorly. In particular, *derriere* may play a role in activating posterolateral (paraxial) mesodermal fates at this time, before somite formation. By mid to late gastrula, *derriere* 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-derriere*-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-derriere* 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 *derriere*, *FGF* cannot direct formation of a secondary axis, *derriere* 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- $\beta$  pathway and these pathways interact.

Why has the requirement for TGF- $\beta$  signaling in formation of posterior structures previously been missed? One possibility is that *Derriere* is the only TGF- $\beta$  ligand specifically required for posterior determination and this requirement could only have been picked up by specifically inhibiting *derriere*. Since a general inhibition of TGF- $\beta$  signaling in *Xenopus* prevents all mesoderm formation, resulting in anaxial embryos (Hemmati-Brivanlou and Melton, 1992), a later posterior requirement for TGF- $\beta$  signaling would have been masked. Second, *Derriere* 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 *Derriere* pathway might also not be active.

### A model for *derriere* function

Our data suggest a model for *derriere* function (Fig. 10). Maternal *VegT* activates expression of *derriere* soon after the mid-blastula transition. Soon after this, a regulatory loop is established between *derriere*, zygotically expressed *VegT*, *eFGF/FGF9* and possibly *Xbra*. This loop maintains

expression of *derriere* in the marginal zone, but fails to maintain expression vegetally, perhaps because of a lack of vegetal FGF signaling (Cornell et al., 1995). Dorsally, *derriere* expression is highest due to the activity of dorsal-specific factors. At early gastrula, *Derriere*, along with other factors, may induce a dorsoventral array of mesodermal genes in the marginal zone. As *derriere* 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 *derriere* RNA is not expressed in the somites, it must either activate muscle formation during gastrulation or *Derriere* protein must persist after its RNA has disappeared. We speculate that *derriere* 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 *derriere* and *FGF*. However, *derriere* 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- $\beta$  family member, *Derriere*. Future directions include analyzing the regulatory loop between *FGF*, *VegT* and *derriere* suggested by these studies, and addressing further the mechanism by which *derriere* regulates posterior patterning.

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