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Effects of heterodimerization and proteolytic processing on Derrière and Nodal activity: implications for mesoderm induction in *Xenopus*

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SUMMARY

Derrière is a recently discovered member of the TGFβ superfamily that can induce mesoderm in explant assays and is expressed at the right time and location to mediate mesoderm induction in response to VegT during *Xenopus* embryogenesis. We show that the ability of Derrière to induce dorsal or ventral mesoderm depends strictly on the location of expression and that a dominant-negative Derrière cleavage mutant completely blocks all mesoderm formation when ectopically expressed. This differs from the activity of similar Xnr2 cleavage mutant constructs, which are secreted and retain signaling activity. Additional analysis of mesoderm induction by Derrière and members of the Nodal family indicates that these molecules are involved in a mutual positive-feedback loop and antagonism of either one of the signals can reduce the other. Interaction between Derrière and members of the Nodal family is also shown to occur through the formation of heterodimeric ligands. Using an oocyte expression system we show direct interaction between the mature Derrière ligand and members of both the Nodal and BMP families. Taken together, these findings indicate that Derrière and Nodal proteins probably work cooperatively to induce mesoderm throughout the marginal zone during early *Xenopus* development.

Key words: BMP, Bone morphogenetic protein, Derrière, Nodal, Xnr, Proteolytic processing, Cerberus, Dominant negative, Heterodimer, Mesoderm induction, *Xenopus laevis*

INTRODUCTION

Induction of the mesoderm takes place prior to gastrulation and represents the earliest known cell-cell interaction in the *Xenopus laevis* embryo. Several secreted growth factors from the FGF and TGFβ families have been identified as candidate mesoderm inducers based on their expression patterns and their ability to convert ectoderm to mesodermal fates (reviewed by Slack, 1994; Harland and Gerhart, 1997). These candidate molecules include bFGF, eFGF, Activin, Xnr1, Xnr2, Xnr4, Xnr5, Xnr6, Derrière and Vg1 (Kimelman and Kirschner, 1987; Slack et al., 1987; Smith et al., 1990; Thomsen et al., 1990; Thomsen and Melton, 1993; Jones et al., 1995; Joseph and Melton, 1997; Sun et al., 1999; Takahashi et al., 2000). Embryos expressing dominant negative Activin type I or type II receptors fail to form mesoderm, indicating that an Activin-like signal is essential for mesoderm formation in *Xenopus* (Hemmati-Brivanlou and Melton, 1992; Chang et al., 1997).

Recent work has shown that the cascade leading to mesoderm induction is initiated by the localized determinant VegT (reviewed by Kimelman and Griffin, 2000). VegT (also known as Brat and Xombi; the zygotic isoform is known as Antipodean) is a maternally supplied T-box transcription factor whose mRNA is stored in the vegetal cortex of the *Xenopus* egg (Lustig et al., 1996; Stennard et al., 1996; Zhang and King, 1996; Horb and Thomsen, 1997). It is essential for the mesoderm-inducing activity of endoderm, and the amount of mesoderm generated by *Xenopus* embryos depleted of maternal *vegT* transcripts is reduced by up to 90% (Zhang et al., 1998; Kofron et al., 1999). VegT promotes *derrière*, *xnr4* and *activin βB* expression in a cell-autonomous manner (Clements et al., 1999; Kofron et al., 1999; Sun et al., 1999; Yasuo and Lemaire, 1999), showing that these mesoderm-inducing signals in *Xenopus* are a target of VegT. Although many mesoderm-inducing signal candidates have been identified, the importance of each and the degree to which they act redundantly has not been fully addressed.

Numerous studies in *Xenopus* have implicated Nodal-related factors (Xnrs) in mesoderm formation (reviewed by Schier and Shen, 2000). Six Xnrs have been identified to date and all but Xnr3 exhibit potent mesoderm-inducing activity (Jones et al., 1995; Smith et al., 1995; Joseph and Melton, 1997; Takahashi et al., 2000). In VegT-depleted embryos, Xnr1, 2 and 4 can all rescue the formation of head, trunk, and tail mesoderm (Kofron et al., 1999). All Xnr proteins characterized to date are initially expressed as zygotic transcripts following mid-blastula transition (MBT) in the endoderm and mesoderm. Transcripts accumulate in a dorsal to ventral gradient, with highest levels seen in dorsal cells (Jones et al., 1995; Agius et al., 2000; Takahashi et al., 2000). The strongest evidence that Nodal signaling is required in *Xenopus* mesoderm formation comes from studies exploiting the short form of the secreted

extracellular antagonist Cerberus (Cer-S). While full length Cerberus is a multifunctional antagonist of Nodal, BMP and Wnt signaling (Bouwmeester et al., 1996; Hsu et al., 1998; Piccolo et al., 1999), the C-terminal Cer-S fragment lacks anti-BMP and anti-Wnt activities and has been suggested to be a Nodal-specific inhibitor (Piccolo et al., 1999). Cer-S blocks mesoderm induction by endodermal explants and inhibits mesoderm formation in whole embryos (Agius et al., 2000). It is difficult to fully reconcile the effects of Cer-S with those of a dominant negative Xnr2 cleavage mutant construct (CM-Xnr2), which has also been described as a specific antagonist of Nodal signaling. While CM-Xnr2 leads to anterior truncations in *Xenopus*, it fails to block mesoderm formation either in embryos or explants (Osada and Wright, 1999).

Findings in zebrafish and mice confirm that Nodal proteins constitute an important part of the mesoderm-inducing signal but indicate that other factors are probably involved, especially in posterior mesoderm formation. Mice homozygous for a *Nodal* mutation have severe defects in mesoderm formation. However, a significant fraction of *Nodal*$^{-/-}$ embryos still express molecular markers of nascent and posterior mesoderm (Conlon et al., 1994). Zebrafish mutant for the *Nodal*-related genes *cyclops* and *squint* fail to express the dorsal mesoderm marker *goosecoid* and lack dorsal expression of the mesodermal marker *ntl*/*brachyury*. At later stages, the only mesodermal derivatives detected are several somites in the tail (Feldman et al., 1998). Loss of *one-eyed pinhead* (*oep*), which encodes an essential extracellular cofactor for Nodal signaling, leads to an almost identical phenotype (Gritsman et al., 1999; Yeo and Whitman, 2001). Similarly, mice mutant for cripto (a murine homologue of *oep*) have severe defects in mesoderm formation, with brachyury expression mislocalized proximally and a complete loss of later markers of axial, paraxial and lateral plate mesoderm (Ding et al., 1998).

These results suggest that while Nodal proteins are an essential component of the mesoderm-inducing signal in vertebrate embryos, other TGFβs probably play a significant role. One likely candidate is Derrière, a TGFβ family member closely related to Vg1. In *Xenopus*, *derrière* is first expressed following MBT in a pattern reminiscent of *vegT*. In animal cap explants, *derrière* transcripts are induced in response to VegT and *derrière* can induce both its own expression and that of *vegT*, suggesting an autoregulatory feedback loop. Although *derrière* has been implicated in posterior development and left-right asymmetry, it can efficiently induce both dorsal and ventral mesodermal fates in animal caps (Sun et al., 1999; Hanafusa et al., 2000). Finally, a dominant negative *derrière* cleavage mutant (*cm-der*) has been shown to block the ability of *vegT* to rescue endodermal gene expression and blastopore formation in *vegT* depleted *Xenopus* embryos (Xanthos et al., 2001).

To evaluate the contribution of TGFβ family members other than Nodal proteins to mesoderm induction, we have undertaken an analysis of *derrière* activity in the early *Xenopus* embryo. We have compared the early embryonic expression pattern of *derrière* with other likely components of the mesoderm-inducing signal and have characterized the activity of *derrière* in the gastrula stage embryo. We have also analyzed the effect of CM-Der on mesoderm induction and find that it completely inhibits the formation of both dorsal and ventral mesoderm. Interference with Derrière function also affects the transcriptional feedback loop previously documented for Nodal and Derrière expression (Takahashi et al., 2000; Onuma et al., 2002) Our analysis of the role of Derrière in mesoderm induction has led to several surprising observations. First, we find that not all TGFβ cleavage mutants act as expected, with unprocessed forms Xnr2 retaining long range mesoderm inducing activity. We also find that Derrière can directly interact with Nodal proteins and BMPs through the formation of heterodimeric ligands. We present our analysis of Derrière function and its interaction with other members of the TGFβ superfamily.

MATERIALS AND METHODS

Whole-mount in situ hybridization and lineage tracing

General methods are described in Sive et al. (Sive et al., 2000). For comparison of *derrière* expression with other mesoderm- and endoderm-specific transcripts, embryos were allowed to develop to the indicated stages and fixed for 1 hour. A subset of the fixed embryos were then bisected prior to in situ hybridization using a razor blade. Gastrula stage embryos were oriented such that the plane of section passed through the dorsal blastopore lip. For lineage tracing, 200 pg of *nuclear β-galactosidase* mRNA was co-injected and visualized following fixation (Sive et al., 2000) with the modification that 6-chloro-3-indolyl-β-D-galactoside (red-gal; Research Organics) was used in place of X-gal.

Construction of Xnr2 cleavage mutants

The general strategy for generating TGFβ cleavage mutants has been described previously (Lopez et al., 1992; Hawley et al., 1995). The original Xnr2 cleavage mutant (CM-Xnr2) was a gift from Christopher V. E. Wright (Osada and Wright, 1999). Additional Xnr2 cleavage mutant constructs were made using CM-*xnr2* in pCS2(+) vector as a template. To generate the Xnr2 double cleavage mutant (DCM-Xnr2), the putative cryptic proteolytic cleavage site RGVR (AGA GGA GTA AGG) at residues 209-212 was altered to ALDA (GCT CTA GAC GCA) by PCR. An upstream cleavage mutant (UCM-Xnr2), in which the cryptic proteolytic cleavage site is mutated but the canonical site is not, was created by replacing the upstream region of wild-type Xnr2 with the equivalent region of DCM-Xnr2 in pCS2 by *Dra*III restriction digest.

To generate the Xnr2 mature domain with an altered proteolytic cleavage site fused to the Activin pro domain (proAct-CM-Xnr2), the coding region of the original CM-*xnr2* construct was first inserted into pCS107 (Baker et al., 1999) and the *xnr2* pro domain was excised using the 5′ *Bst*BI restriction site and a *Sal*I site introduced into the altered cleavage site. The pro domain of *activin βB* was amplified from pCSKA Activin βB plasmid (Thomsen et al., 1990) by high fidelity PCR and inserted into the *Bst*BI/*Sal*I pCS107 CM-Xnr2 vector. All PCR reactions used high fidelity *Pfu* polymerase (Stratagene) and the final constructs were checked for alterations both by sequencing and whole embryo activity assay.

Construction of epitope tagged Derrière, Xnr2 and CM-Derrière

HA- and Flag-tagged *proAct-xnr2* and *proAct-derrière* constructs were made with the *activin βB* pro domain in place of the wild-type pro domain to increase proteolytic processing efficiency. The HA-tagged constructs used the pCS2(+) proAct-HA-Xnr1 cassette vector described by Piccolo et al. (Piccolo et al., 1999). The *xnr1* mature domain was excised using a *Xho*I restriction site immediately downstream of the HA-tag and a 3′ *Xba*I site. The mature domains of *xnr2* and *derrière* were amplified by high fidelity PCR and cloned into the proAct-HA cassette.

To generate the equivalent Flag-tagged constructs, a Flag epitope containing a 5′ *Sal*I site and a 3′ *Xho*I site was introduced at an endogenous *Xho*I restriction site immediately downstream from the proteolytic cleavage sequence in *activin βB* in pSP64T (Thomsen et al., 1990). The resultant construct contains the sequence RIRKRGL-(DYKDDDDK)LE (Activin proteolytic cleavage site underlined; Flag-tag in parentheses) and includes an *Xho*I restriction site following the Flag epitope. The mature domains of *xnr2* and *derrière* were amplified by PCR as previously described, and cloned into the *Xho*I/*Xba*I proAct-Flag cassette to create the proAct-Flag-Xnr2 and proAct-Flag-Derrière constructs.

Wild-type Derrière and Xnr2 proteins, which possess endogenous pro and mature domains, were also tagged with Flag and HA epitopes. To generate these constructs, the *derrière*- and *xnr2*-coding regions were subcloned into pCS108 (A gift from Mustafa Khoka, UC Berkeley) and a *Sal*I site was introduced by PCR mutagenesis immediately downstream of the proteolytic cleavage site. Sequences encoding either the Flag or HA epitope and containing a 5′ *Sal*I site and a 3′ *Xho*I site were then inserted at the *Sal*I restriction site. The resultant constructs are Flag-Derrière: RAKRV(DYKDDDDK)LD; HA-Derrière: RAKRVD(YPYDVPDYA)LD; HA-Xnr2: RRPRRVD-(YPYDVPDYA)LD (proteolytic cleavage sites underlined; epitope tags in parentheses).

The cleavage mutant form of *derrière* (CM-*der*) (Sun et al., 1999) was cloned into pCS107 (Baker et al., 1999) and a single copy of the Glu-Glu epitope inserted at a *Sal*I restriction site in the altered proteolytic cleavage sequence. The introduction of the Glu-Glu-tag changed the altered cleavage site from GVDG to GVD(GE-YMPMEG)VDG (Glu-Glu-tag in parentheses). As with the Xnr2 cleavage mutant constructs, all epitope tagged constructs were checked by sequencing and activity assay for the presence of PCR induced mutations.

Synthesis and microinjection of synthetic mRNA

Capped synthetic mRNAs were transcribed using the mMessage mMachine kit (Ambion). The open reading frames of *Xenopus derrière* and CM-*der* (Sun et al., 1999) were amplified by high fidelity PCR and cloned into *Eco*RI/*Xho*I digested pCS107. The creation of other constructs is described above. *Xenopus derrière*, CM-*der* and Glu-Glu-tagged CM-*der* mRNAs were made from pCS107 (Baker et al., 1999) vectors linearized with *Asc*I and transcribed by SP6 RNA polymerase. *cerberus short-form* (*cer-S*) (Piccolo et al., 1999), *proact-HA-der*, and *proact-HA-xnr2* mRNAs were made from pCS2(+) linearized with *Not*I and transcribed by SP6. *bmp4-flag* (a gift from Jan L. Christian, Oregon Health Sciences University), CM-*xnr2*, DCM-*xnr2* and *proact*-CM-*xnr2* mRNAs were made from pCS2(+) linearized with *Asp*718 and transcribed with SP6. *Proact-flag-der*, *proact-flag-xnr2* and *proact-xnr2* were made from pSP64T (Krieg and Melton, 1984) linearized with *Eco*RI and transcribed with SP6. *Nuclear β-galactosidase* mRNA was made from pSPnucβGal (Smith and Harland, 1992) linearized with *Xho*I and transcribed by SP6. All mRNAs were injected as indicated.

Animal cap assays and RT-PCR

For animal cap assays, one-cell embryos were injected with synthetic mRNAs as indicated at the animal pole. Animal cap ectoderm was dissected at stage 8 and cultured in 75% NAM solution (Peng, 1991). RNA was harvested at stages 11 (Condie and Harland, 1987) and 20 and RT-PCR was performed as described by Wilson and Melton (Wilson and Melton, 1994). Primer sets and amplification conditions have been described for *ef1α*, *xbra*, *gsc*, *muscle actin* and *n-cam* (Wilson and Melton, 1994); *chordin* (Sasai et al., 1994); *odc* (Hudson et al., 1997); *cerberus* and *nrp-1* (Mariani and Harland, 1998); *vegT* (Zhang et al., 1998); *xnr1* and *xnr2* (Kofron et al., 1999); and *xvex1* (Shapira et al., 1999). Primers used for *derrière* were U, 5′-TGG TTA CAT GGC AAA CTA CTG C-3′ (nucleotide positions 825-846); D, 5′-GAA TGA GAA CAA TCT CCA AAG C-3′ (nucleotide positions 1070-1091; note that the open reading frame encoding Derrière stops at nucleotide 1065, placing the downstream primer in the 3′ UTR and allowing for selective amplification of endogenous *derrière* mRNA). Other primer sequences were obtained from http://cbrmed.ucalgary.ca/pvize/html/WWW/Marker_pages/primers.html. For all RT-PCR reactions either *ef1α* or *odc* were used as loading controls.

Preparation of secreted proteins from oocytes

Secreted [^{35}S]-labeled proteins were prepared from *Xenopus* oocytes essentially as described (Sive et al., 2000). For expression of single proteins, 40 ng of synthetic mRNA was injected per oocyte; for co-expression of two proteins, both mRNAs were mixed at equimolar concentrations and a total of 80 ng was injected. Conditioned medium was stored at 4°C. To examine intracellular proteins, oocytes were lysed in ice-cold RIPA buffer (20 μl/oocyte) (Harlow and Lane, 1998) and centrifuged twice for 15 minutes at 4°C at 13,000 ***g*** to remove the yolk. After each round of centrifugation, the clear middle layer was retained.

Immunoprecipitations

Supernatants and oocyte lysates were immunoprecipitated using the indicated antibodies; all immunoprecipitations used two to five oocyte equivalents (20-50 μl) of either supernatant or lysate. αGlu-Glu (clone Glu-Glu; Covance, Richmond, CA) and αFlag (clone M2; Sigma) immunoprecipitations were carried out in a mixture of 60% RIPA and 40% phosphate-buffered saline (PBS) with the antibody at 2 ng/μl. Anti-HA immunoprecipitations were carried out in 60% RIPA and 40% αHA mouse monoclonal antibody (clone 12CA5) hybridoma tissue culture supernatant. Binding was carried out on ice for 1 hour in a total volume of 500 μl. Antibodies were then bound to protein A beads for 30 minutes at 4°C with end-over-end rotation, centrifuged and washed three times in RIPA buffer. For two-step immunoprecipitations, the αHA monoclonal antibody was first coupled to protein A beads (Harlow and Lane, 1988). Bound proteins were released by boiling for 10 minutes in Laemmli sample buffer. For all immunoprecipitations, immunoprecipitating proteins were resolved by 12% reducing SDS-PAGE.

RESULTS

The expression pattern of *derrière* during early *Xenopus* development suggests a role in mesoderm induction

Although Nodal proteins have been implicated as a major component of the mesoderm-inducing signal in *Xenopus*, zebrafish and mouse, there is reason to believe that other members of the TGFβ family also play a crucial role. In *Xenopus* embryos, *derrière* is a likely candidate for contributing to mesoderm formation based on its activity in naïve ectodermal explants and its expression pattern (Sun et al., 1999). To better understand the role *derrière* may play in mesoderm induction, we undertook a thorough analysis of its spatial and temporal expression pattern during blastula and gastrula stages.

The timing and distribution of *derrière* transcripts were compared with well-characterized tissue-specific markers and other members of the TGFβ family implicated in mesoderm formation. The temporal expression of *derrière* was analyzed by RT-PCR on RNA from whole embryos. As previously reported (Sun et al., 1999), *derrière* is present only as a zygotic transcript and cannot be detected prior to MBT at stage 8 (Fig. 1A, lanes 1 and 2). Transcripts are first detected at stage 8.5 and rapidly accumulate (Fig. 1A, lanes 3 and 4). Levels plateau

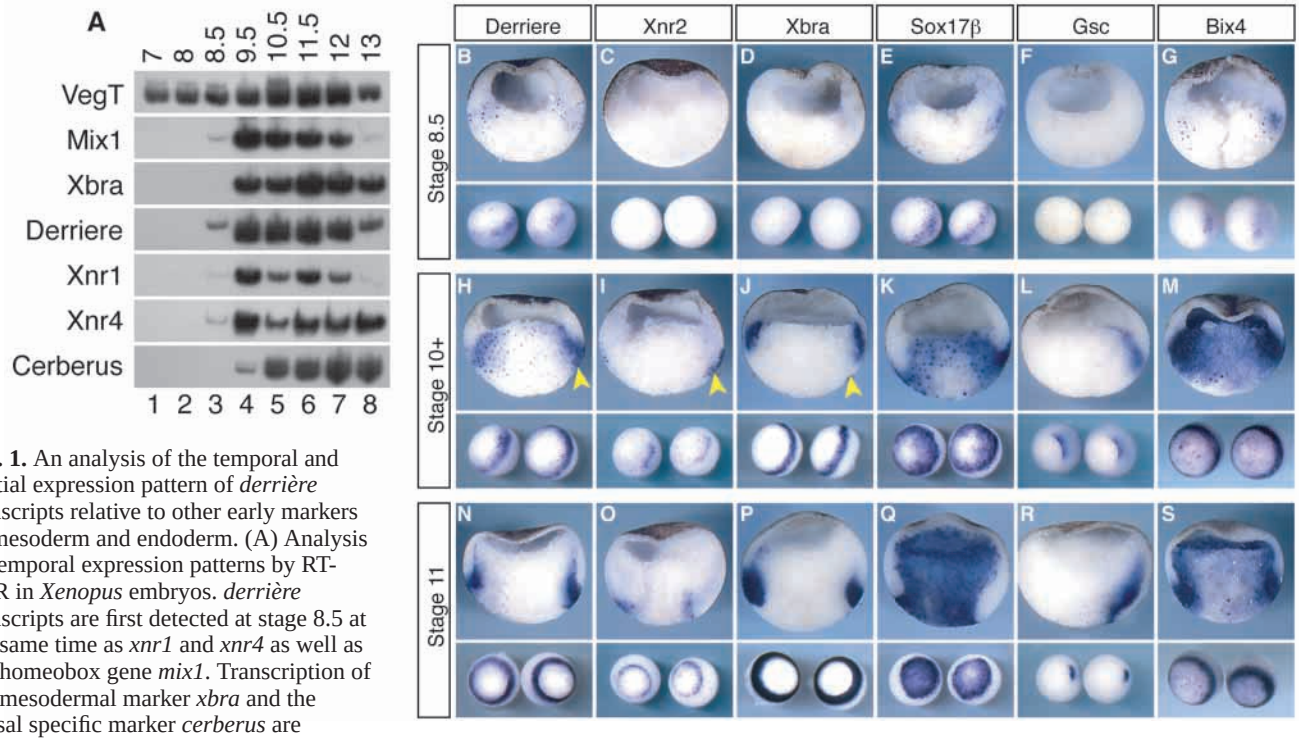

Fig. 1. An analysis of the temporal and spatial expression pattern of *derrière* transcripts relative to other early markers of mesoderm and endoderm. (A) Analysis of temporal expression patterns by RT-PCR in *Xenopus* embryos. *derrière* transcripts are first detected at stage 8.5 at the same time as *xnr1* and *xnr4* as well as the homeobox gene *mix1*. Transcription of the mesodermal marker *xbra* and the dorsal specific marker *cerberus* are detected soon afterwards. (B-S) Whole-mount in situ hybridization analysis of *Xenopus* embryos at stages 8.5, 10+ and 11. The upper panels in each row show a representative embryo bisected through the animal-vegetal axis and oriented with the dorsal side on the right. The lower panels show a vegetal view of whole embryos, again oriented with dorsal sides to the right. (B-G) *derrière*, *sox17β* and *bix4* transcripts are detected in distinct but overlapping domains at stage 8.5. Localized expression of *xnr2*, *xbra* and *gsc* is not apparent at this stage. (H-M) All transcripts show strong localized expression by stage 10+. *derrière* expression mirrors that of *xbra*, while *xnr2* transcripts are restricted to the superficial cells of the marginal zone and greatly enriched on the dorsal side. (N-S) Expression in stage 11 embryos. Again *derrière* transcripts are detected throughout the region of the embryo expressing *xbra*. Arrowheads indicate the location of the dorsal blastopore lip in stage 10+ embryos.

by stage 9.5 and remain high throughout gastrulation, but taper off by stage 13 (Fig. 1A, lanes 5-8). The onset of *derrière* expression coincides with the onset of other presumptive targets of the maternally supplied transcription factor VegT. These include the homeobox genes *mix1* (Fig. 1A) (Clements et al., 1999) and *bix4* (Fig. 1G) (Tada et al., 1998; Casey et al., 1999) as well as *xnr1* and *xnr4* (Fig. 1A). Expression of the mesodermal marker *xbra* is not detected until stage 9.5 (Fig. 1A, lane 4), as expected for a gene whose activation depends on one or more of the zygotically supplied TGFβs. Expression of *cerberus*, in the dorsal-most region of the mesendoderm (Bouwmeester et al., 1996; Zorn et al., 1999), is also first detected at stage 9.5 and increases to maximum levels during early gastrula stages (Fig. 1A, lane 5) as dorsoventral patterning of the mesoderm commences.

In order to compare the spatial expression pattern of *derrière* with other TGFβ family members and determine its position relative to the boundaries of the three germ layers, we analyzed embryos by whole-mount in situ hybridization. Embryos were bisected prior to in situ hybridization to reveal expression patterns in deep cells. In agreement with RT-PCR analysis, *derrière* transcripts are detected at stage 8.5 (Fig. 1B). The entire marginal zone expresses *derrière*, both superficially in cells of the suprablastoporal endoderm and throughout the underlying mesoderm all the way to the blastocoel. In addition, expression is detected in the vegetal cells of the sub-blastoporal endoderm. Although *derrière* appears to exhibit a slight dorsal bias, expression is clearly detected throughout the marginal zone. At this stage, *derrière* expression is highly reminiscent of that of *bix4*, a direct target of VegT (Fig. 1G). The HMG-domain transcription factor *sox17β* is expressed by stage 8.5, but is limited to the vegetal endoderm and superficial cells of the marginal zone (Fig. 1E) [see Henry and Melton (Henry and Melton, 1998), and Zorn et al. (Zorn et al., 1999) for additional analysis of endoderm and mesoderm markers in sectioned embryos]. Expression of mesodermal markers such as *xbra* and *gsc* is not yet seen at this stage (Fig. 1D,F).

At gastrulation stages 10+ and 11, *derrière* is strongly expressed throughout much of the marginal zone and in at least some cells of the sub-blastoporal endoderm (Fig. 1H,N). Its expression pattern continues to closely mirror that of *bix4* (Fig. 1M,S). Localized transcription of *xnr2* is apparent by stage 10+ and shows a strong dorsal bias (Fig. 1I). Relative to *derrière*, which is expressed at high levels in both the superficial and underlying cells of the mesoderm and endoderm, *xnr2* is clearly restricted to the surface of the embryo and appears to be most closely associated with the blastopore lip (Jones et al., 1995). Expression of *xnr1*, *xnr5* and *xnr6* is not as restricted to the embryonic surface as is *xnr2*, but all show the same strong dorsal bias, which probably reflects positive regulation by the dorsal determinant β-catenin (Agius et al., 2000; Takahashi et al., 2000). *derrière* is expressed more broadly than the mesodermal marker *xbra* (Fig. 1J,P), particularly at the onset of gastrulation. At stage 10+, the high levels of *derrière* transcript seen in the marginal zone extend vegetally all the way to the dorsal blastopore lip (Fig. 1H; arrowhead indicates

blastopore lip). By contrast, *xbra* expression terminates several cell diameters above the blastopore lip (Kumano and Smith, 2000). In summary, *derrière* expression corresponds to what is expected for an endogenous mesoderm-inducing signal: it is initiated prior to the onset of mesoderm formation and is present at high levels in both the dorsal and ventral marginal zone, and in the underlying cells of the vegetal endoderm.

derrière can induce both dorsal and ventral mesoderm

derrière has been previously shown to induce a number of mesodermal markers in animal cap assays and to induce a partial secondary axis when injected ventrally (Sun et al., 1999). To understand the capacity of *derrière* to induce mesoderm in the context of the whole embryo further, we overexpressed *derrière* mRNA in either the dorsal or ventral marginal zone. Although even modest doses of *xnrs* and *activin* strongly induce dorsal mesoderm and convert ventral fates to dorsal, we found that *derriere* was much less potent as a dorsal inducer. Both dorsal and ventral injections induce general mesoderm markers such as *xbra* (Fig. 2B,C). However, dorsal targeting leads to an expansion of dorsal markers such as *gsc* (Fig. 2E) while ventral targeting leads to an expansion of ventral and lateral markers such as *xvent1* (Fig. 2I). The ability of *derrière* to induce ventrolateral fates in addition to dorsal ones is consistent with the loss of anterior structures reported by Sun et al. (Sun et al., 1999). Head formation requires the suppression of trunk fates, so that an expansion of trunk mesoderm is expected to inhibit head formation. Unlike *xnr2*, which will strongly induce organizer fates on the ventral side at only 10 pg (P. M. E. and R. M. H., unpublished), *derrière* does not induce ectopic *gsc* expression even at 200 pg (Fig. 2F). *derrière's* reported ability to induce partial secondary axes when overexpressed ventrally (Sun et al., 1999) could result from the induction of ventrolateral rather than organizer fates.

Dominant-negative *derrière* completely inhibits mesoderm formation in *Xenopus*

To determine if *derrière* is an essential component of the endogenous mesoderm inducing signal in *Xenopus*, we used a dominant-negative approach to analyze loss of function. As TGFβs must be proteolytically cleaved after dimerization to form an active ligand, a non-cleavable version can dimerize with endogenous wild-type monomers and prevent proper processing (Fig. 3K) (Lopez et al., 1992). A *derrière* cleavage mutant (CM-*der*) has previously been described by Sun et al. (Sun et al., 1999). We injected 2 ng of CM-*der* mRNA into the marginal zone of a single blastomere at the four-cell stage. This dose of CM-*der* is enough to inhibit mesoderm induction in animal caps by up to 200 pg of *derrière* (Fig. 3L, lane 3). Embryos were allowed to develop to mid-gastrula stages and fixed for whole-mount in situ hybridization analysis. The site of mRNA injection was identified by detection of a *β-galactosidase* lineage tracer. In embryos where CM-*der* had been targeted to the marginal zone, expression of the general mesodermal marker *xbra* was completely lost at the site of injection (Fig. 3B). In addition, the blastopore lip either failed to form in the presence of CM-*der* or was severely disorganized (not shown). The complete inhibition of mesoderm formation caused by CM-*der* is highly reminiscent of the activity reported for *cer-S* (Fig. 3D) (Agius et al., 2000) and is consistent with the loss of trunk mesoderm reported by Sun et al. (Sun et al., 1999).

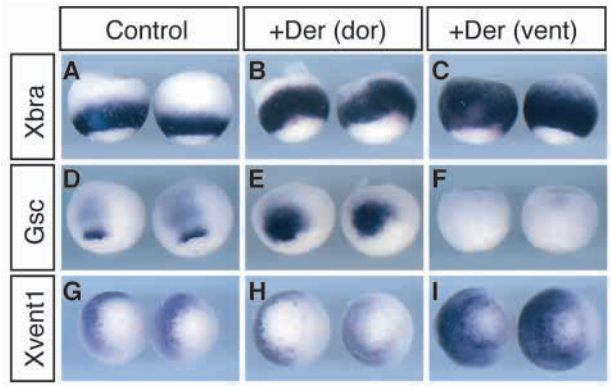

Fig. 2. *derrière* induces both dorsal and ventral markers of mesoderm in whole embryos. Embryos were injected with 200 pg of *derrière* (*der*) mRNA into a single blastomere at the four-cell stage. Injections were targeted to either the dorsal (dor) or ventral (vent) marginal zone and gastrula stage embryos were analyzed by whole-mount in situ hybridization. (A-F) Embryos viewed from the side of injection. (A-C) Expression of the mesodermal marker *xbra* is induced on the side of injection. (D-F) Dorsal injection leads to expanded *gsc* expression, while ventral injection causes no ectopic expression of *gsc* in the ventral marginal zone. (G-I) Embryos viewed from the vegetal pole with dorsal facing rightwards. Ventral injection of *derrière* mRNA leads to increased expression of the ventral and lateral marker *xvent1*, while dorsal injection has no effect.

As *derrière* and *nodal* genes are linked to mesoderm induction both by their expression patterns and activity, we reasoned that their transcription might be inhibited when mesoderm is blocked by CM-*der* and *cer-S*. This was borne out in both animal cap experiments and whole embryos expressing *cer-S*. Animal caps co-injected with *derrière* and *cer-S* failed to express *xbra*, d*erriere* and *xnr1* (Fig. 3L, lane 4). In situ hybridization analysis of embryos injected marginally with *cer-S* mRNA also revealed a substantial decrease in the expression of both *derrière* and *xnr2* at stage 11 (Fig. 3G,J). In addition to being significantly reduced in level, the boundaries of the strong band of *derrière* expression normally seen at the marginal zone were much less distinct than in control embryos and more punctate expression was detected in the vegetal endoderm (compare expression in Fig. 3E,G). Injection of CM-*der* prevents analysis of endogenous *derrière*, but CM-*der* appeared to cause a modest reduction in *xnr2* transcription (Fig. 3I). These data demonstrate that Derrière and Nodal proteins positively regulate each other at the transcriptional level during mesoderm induction; therefore even reagents that block only one of these molecules through direct interaction (such as Cer-S) still have inhibitory effects on both.

Xnr2 retains diminished signaling capacity even when it is unable to be proteolytically processed

When a cleavage mutant form of *xnr2* (CM-*xnr2*) (Osada et al., 1999) was compared with CM-*der* and *cer-S*, striking differences in activity were apparent. Expression of *xbra* was greatly expanded and could be seen in most of the animal hemisphere (Fig. 3C). CM-*xnr2* was also found to upregulate *derrière* expression (Fig. 3F). These effects on cells far

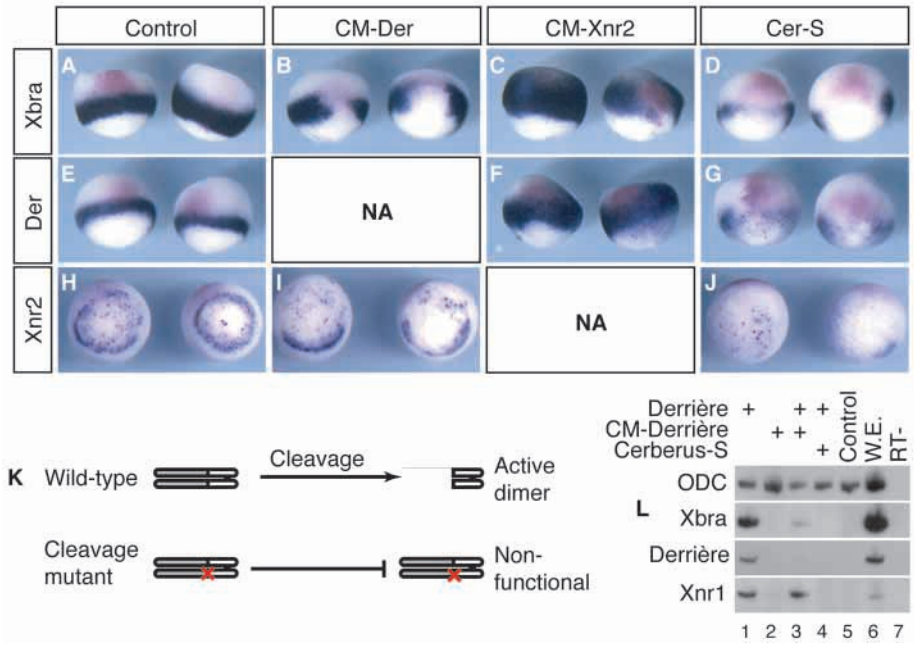

Fig. 3. A dominant negative *derrière* cleavage mutant (CM-*der*) inhibits mesoderm induction in *Xenopus* embryos. The effect of CM-*der* on mesoderm formation is similar to that of the short form of *cerberus* (*cer-S*) but differs markedly from the activity of the *xnr2* cleavage mutant (CM-*xnr2*). Embryos were injected with either 2 ng of CM-*der*, 2 ng of CM-*xnr2*, or 500 pg of *cer-S* mRNA into a single blastomere at the four-cell stage along with 200 pg of a *β-galactosidase* lineage tracer. Stage 11 embryos were stained with red-gal to mark the site of injection and analyzed by whole-mount in situ hybridization. (A-D) Expression of the mesodermal marker *xbra* is inhibited in a similar manner by CM-*der* and *cer-S*; CM-*xnr2* causes expansion of *xbra* into the animal hemisphere. (E-G) Expression of *derrière* is significantly diminished by *cer-S* but is expended into the animal hemisphere by CM-*xnr2*. (H-J) *Xnr2* expression is strongly inhibited by *cer-S* and appears to be partially attenuated in the presence of CM-*der*. In H-J, embryos have been oriented with the site of mRNA injection at the top. (K) Mechanism of inhibition by cleavage mutant constructs. (L) *derrière* activity is blocked by both CM-*der* and *cer-S* in animal caps. Animal poles were injected at the one-cell stage with 200 pg *derrière* mRNA and co-injected with either 2 ng CM-*der* or 500 pg *cer-S*. By itself, *derrière* induces *xbra* and *xnr1*, as well as upregulating its own transcription. CM-*der* shows no mesoderm-inducing activity on its own and significantly reduces mesoderm induction by wild-type *derrière*. *cer-S* also blocks mesoderm formation in animal caps expressing *derrière*.

removed from the site of expression are of particular interest because TGFβ cleavage mutants are conventionally thought to function cell autonomously by blocking secretion as well as proteolytic processing.

The induction of mesoderm-specific transcripts by CM-*xnr2* indicates that it may retain at least some of the signaling activity of wild-type *xnr2*. One possible explanation for this is suggested by the presence of a cryptic proteolytic cleavage in the pro region of Xnr2, upstream from the canonical cleavage site. TGFβs are cleaved by members of the proprotein convertase family of endoproteases that recognize the consensus cleavage site RXXR (Bresnahan et al., 1990; Molloy et al., 1992; Dubois et al., 1995; Cui et al., 1998). Recent work has shown that the TGFβ family member Lefty A is processed at two distinct cleavage sites, each of which retains activity when the other is mutated (Ulloa et al., 2001). In addition, BMP4 is cleaved in an sequential manner at two sites, and mutation of the upstream site has important consequences for both the strength and range of the signal (Cui et al., 2001). In Xnr2, cleavage at both sites would result in two pro domain fragments, which we have designated proA and proB (Fig. 4A). Because only the canonical site was altered in CM-*xnr2*, we reasoned that processing might still be occurring at a cryptic site and generating active ligand consisting of proB and the mature domain. To test this possibility, we altered the second cleavage site at residues 209-212 from RGVR to ALDA, creating a double cleavage mutant *xnr2* (DCM-*xnr2*; Fig. 4A). CM-*xnr2* and DCM-*xnr2* were tested for mesoderm inducing activity in animal cap assays. Surprisingly, we found that even DCM-*xnr2* was still capable of inducing *xbra*. The overall activity of both cleavage mutants was substantially attenuated, as indicated by the fact that even at 2 ng they induced less *xbra* than 10 pg of wild-type *xnr2* (Fig. 4B, lanes 1-3). Reduced signaling capacity was confirmed by the failure of CM-*xnr2* and DCM-*xnr2* to induce extreme dorsal mesoderm. *Xnr2* is capable of inducing a range of mesodermal fates in a concentration-dependent manner, from ventral and lateral derivatives at very low doses to exclusively dorsal fates at higher doses (Jones et al., 1995). While 10 pg of wild-type *xnr2* was sufficient to activate expression of the dorsal marker *gsc* in animal caps, even 2 ng of the cleavage mutants failed to do so. Taken together, these data indicate that unprocessed Xnr2 can transduce a Nodal-like signal and cleavage mutant forms of the protein should not be considered authentic dominant negatives. In contrast to Xnr2, we found that CM-Der is completely inactive in mesoderm induction assays (Fig. 4B, lane 4).

In order to assess the importance of the Xnr2 cryptic cleavage site for normal Nodal signaling, we constructed an upstream cleavage mutant (UCM-Xnr2) in which the cryptic site is mutated while the canonical site is left unaltered. The activity of each was evaluated over a range of doses in both animal caps and whole embryos. In animal cap assays, both wild-type Xnr2 and UCM-Xnr2 were found to induce mesodermal markers such as *xbra* and *vegT* at similar doses (Fig. 5A). When overexpressed in whole embryos both molecules gave rise to identical phenotypes at equivalent doses (Fig. 5B-E). We conclude that proteolytic processing at the canonical cleavage recognition sequence can occur in the absence of upstream processing and is sufficient for normal (or nearly normal) activity.

Because CM-Xnr2 had previously been reported to lack all wild-type activity, including the ability to induce mesoderm (Osada and Wright, 1999), it was important to confirm our

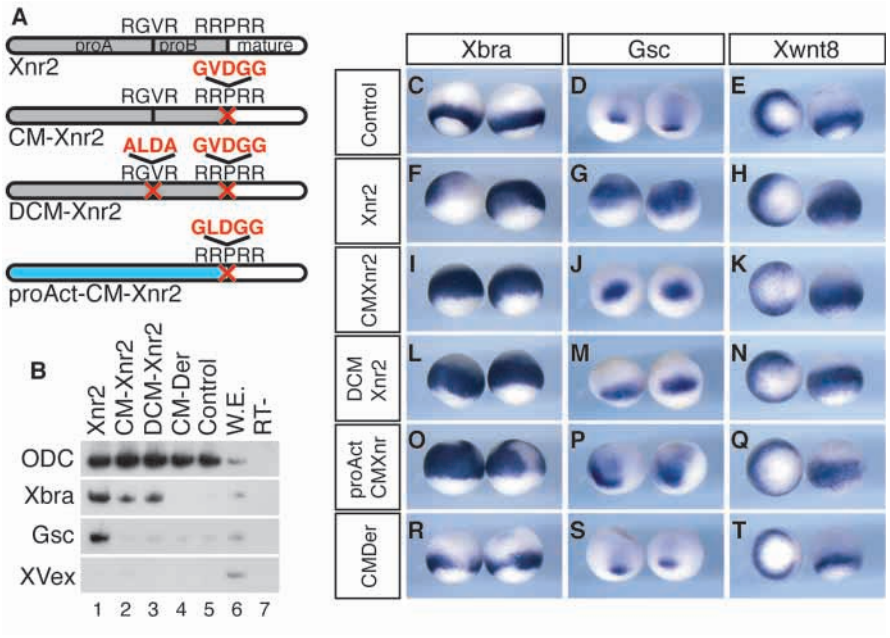

Fig. 4. Cleavage mutant forms of Xnr2 retain diminished signaling activity and therefore do not function as authentic dominant negative molecules. (A) Diagram of the various Xnr2 cleavage mutant constructs tested. The gray region of the bar indicates the Xnr2 proA and proB regions, while the white regions represent the mature Xnr2 ligand. The Activin pro domain is indicated in blue. (B) RT-PCR analysis of *xnr2* cleavage mutant constructs. Embryos were injected in the animal pole at the one-cell stage with 10 pg of *xnr2* or 2 ng of the cleavage mutant mRNAs. Wild-type *xnr2* and both CM-*xnr2* and DCM-*xnr2* induce mesoderm in animal caps, as indicated by the presence of *xbra* transcripts. An equivalent dose of CM-*der* shows no mesoderm inducing activity. Both *xnr2* cleavage mutant constructs fail to induce extreme dorsal fates (marked by *gsc*). (C-T) *xnr2* cleavage mutant constructs induce ectopic mesoderm in whole embryos. Embryos were injected with the 10 pg *xnr2* or 2 ng cleavage mutant mRNAs in the animal pole at the one-cell stage, allowed to develop to stage 11 and analyzed by in situ hybridization. Wild-type *xnr2* and all three cleavage mutant constructs cause expansion of the mesodermal marker *xbra*, the dorsal mesodermal marker *gsc* and the ventral/lateral mesodermal marker *xwnt8* (F-Q). By contrast, CM-*der* leads to an inhibition of *xbra* expression and has no obvious effect on *gsc* and *xwnt8* (R-T), presumably because of limited diffusion of the animally injected mRNA).

observations in additional assays. We therefore made a second attempt to rule out low-level proteolytic processing from any unidentified cleavage sites in the pro region of Xnr2. To do this, we completely replaced the Xnr2 pro region with that of Activin βB, while leaving the cleavage site mutated (proAct-CM-Xnr2) (Fig. 4A, Activin pro region in blue). Previous studies have shown that when the Activin cleavage mutant is translated in *Xenopus* oocytes, only the unprocessed 51 kDa protein is produced (Hawley et al., 1995). This indicates that Activin contains only a single site for proteolytic processing. Consequently, proAct-CM-Xnr2 should be completely inactive if the activity of CM-Xnr2 and DCM-Xnr2 is dependent upon processing from unidentified sites in the pro domain.

We compared the activity of all three Xnr2 cleavage mutants in whole embryos using in situ hybridization to evaluate mesoderm formation. CM-*xnr2* and DCM-*xnr2* both induced *xbra* expression throughout much of the animal hemisphere (Fig. 4I,L). *Proact*-CM-*xnr2* was equally effective at inducing mesoderm in this assay (Fig. 4O). All three cleavage mutant mRNAs, when injected in the animal hemispheres of one-cell embryos, caused expansion of *gsc* on the dorsal side of the embryos (Fig. 4J,M,P) and *xwnt8* (a marker of ventral and lateral mesoderm) in the remainder of the embryos (Fig. 4K,N,Q). Wild-type *xnr2* was more efficient than the cleavage mutants at inducing *gsc* (Fig. 4G), confirming that activity is reduced when proteolytic processing is inhibited. Once again, CM-*der* showed no mesoderm-inducing activity and actually inhibited *xbra* expression in some embryos, despite being targeted to the animal pole rather than the marginal zone (Fig. 4R).

It is possible that Xnr2 cleavage mutants could induce mesoderm either by acting as functional Nodal ligands or by heterodimerizing with and inhibiting endogenous Nodal antagonists such as Antivin and Lefty (Meno et al., 1999;

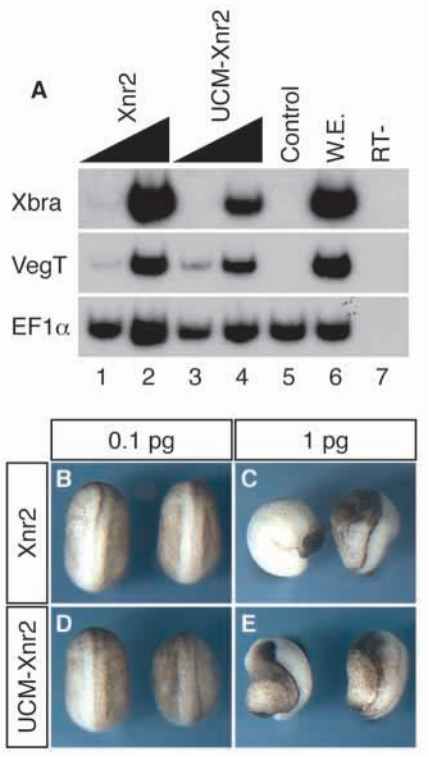

Fig. 5. Loss of the cryptic proteolytic cleavage site in Xnr2 has no impact on activity. (A) RT-PCR analysis of the *xnr2* upstream cleavage mutant construct (UCM-*xnr2*). Embryos were injected in the animal pole at the one-cell stage with 1 pg or 10 pg of *xnr2* or UCM-*xnr2* mRNA. Both molecules induce the mesoderm markers *xbra* and *vegT* at equivalent doses (lanes 1 and 4). (B-E) Analysis of UCM-*xnr2* in whole embryos. Embryos were injected at the one-cell stage with 0.1 pg or 1 pg of *xnr2* or UCM-*xnr2* mRNA and allowed to develop to stage 20.

Thisse and Thisse, 1999; Cheng et al., 2000). In the former case, Xnr2 cleavage mutants would act as secreted mesoderm-inducing signals, while in the later case their activity would be cell autonomous (because even if the unprocessed ligand were to be secreted, heterodimerization with Nodal antagonists could only occur in the expressing cells). To distinguish between these possibilities, we compared the range of DCM-Xnr2 activity with wild-type Xnr2 and the cell-autonomous mesoderm inducer Smad2 (an intracellular component of the Nodal signal transduction pathway). Embryos were injected at the four-cell stage to limit diffusion of mRNAs to one quadrant of the animal hemisphere. To facilitate lineage tracing, a *lacZ/smad2* fusion construct was employed (Baker and Harland, 1996) and *nuclear β-galactosidase* was co-injected with *xnr2* and DCM-*xnr2*. Mesoderm induction was assayed at stage 11 by in situ hybridization. In *smad2*-injected embryos, ectopic *xbra* expression was strictly limited to those cells of the animal hemisphere where Smad2 was also expressed (Fig. 6C). By contrast, both *xnr2* and DCM-*xnr2* caused ectopic *xbra* expression throughout the animal hemisphere even in cells far removed from those injected with mRNA (Fig. 6A,B). While it is possible that antagonism of Antivin and Lefty through heterodimerization accounts for some of the activity seen in the Xnr2 cleavage mutants, these data show that the unprocessed ligand also retains long-range signaling activity.

The ability of CM-Xnr2 and DCM-Xnr2 to act as secreted signaling molecules is unexpected, given that cleavage mutant forms of TGFβs are thought to block secretion of the endogenous, wild-type monomers (Lopez et al., 1992). To confirm that Xnr2 cleavage mutants are secreted, we examined expression of wild-type Xnr2, CM-Xnr2, DCM-Xnr2 and CM-Der proteins in *Xenopus* oocytes. Levels of protein were compared between conditioned oocyte supernatants (containing secreted protein) and lysates (containing intracellular protein). Xnr2, CM-Xnr2 and DCM-Xnr2 were found almost exclusively in the oocyte supernatant, indicating that they are efficiently secreted (Fig. 6E, lanes 1-3). By contrast, CM-Der was not secreted by oocytes and could only be detected in the lysate (Fig. 6E, lane 4). Proteolytic processing was observed in wild-type Xnr2 and CM-Xnr2, indicating that both canonical and cryptic cleavage sites are recognized by proteases. When wild-type Xnr2 was expressed, bands corresponding to both proA and mature domains were observed (Fig. 6E, lane 1; mature ligand indicated by arrowhead). Although a band corresponding to the predicted proB/mature ligand was not observed in CM-Xnr2, the presence of the proA domain indicated proteolytic processing had taken place. As expected, DCM-Xnr2 was present only as an unprocessed precursor protein running at approximately 70 kDa. These results demonstrate that different TGFβ family members have very different activities following proteolytic processing. However, for our purposes, the finding that CM-Der is both inactive and an inhibitor of mesoderm formation enables us to further test its role in normal mesoderm induction.

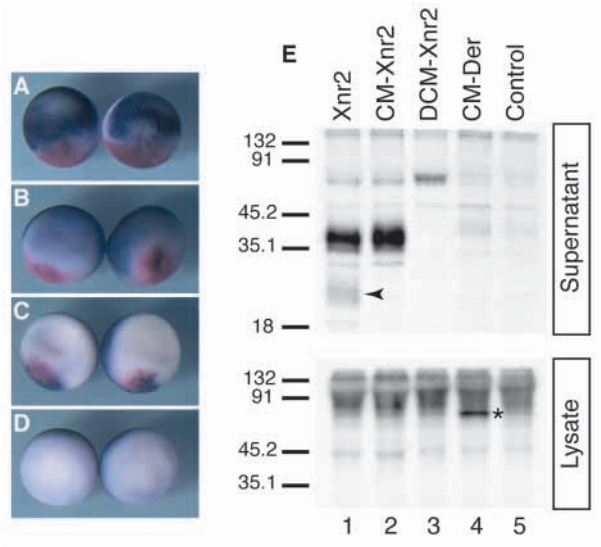

Fig. 6. Cleavage mutant forms of Xnr2 are secreted and can act non-cell autonomously. (A-D) DCM-Xnr2 induces mesoderm non-cell autonomously. Four-cell embryos were injected at the animal pole with (A) 100 pg *xnr2* + 200 pg *β-galactosidase*, (B) 2 ng DCM-*xnr2* + *β-galactosidase* or (C) *lacZ/adr2* mRNA. *lacZ* expression was visualized by red-gal staining and *xbra* expression was detected by in situ hybridization. (D) Control embryos. (E) CM-Xnr2 and DCM-Xnr2 are secreted from oocytes while CM-Der is not. Mature oocytes were injected with 25 ng of the indicated mRNAs and labeled with [^{35}S]methionine. CM-Xnr2 and DCM-Xnr2 are efficiently secreted while CM-Der is retained within the oocyte lysate (asterisk). Both Xnr2 and CM-Xnr2 undergo proteolytic processing, as indicated by the presence of the proB domain (arrowhead). Mutation of both cryptic and canonical sites in DCM-Xnr2 abolishes all proteolytic processing.

Dominant negative Derrière promiscuously antagonizes Nodal signaling

We wanted to evaluate thoroughly the specificity of CM-Der for Derrière signaling. Previous reports have indicated that although CM-Der is a preferential antagonist of Derrière signaling, it may have some effect on other TGFβ family members. In an assay for animal cap elongation (characteristic of dorsal mesoderm fates), CM-*der* was found to reduce both the frequency and extent of *xnr2*-induced elongation (Sun et al., 1999). We confirmed the ability of CM-Der to attenuate Nodal signaling by showing that it can ameliorate the *xnr2* overexpression phenotype in whole embryos (Fig. 7A). Normally, the severity of this phenotype increases with the level of *xnr2* mRNA (for the purposes of this experiment doses ranged from 1 to 10 pg). When *xnr2* is co-injected with 3 ng of CM-*der*, the whole embryo phenotype is markedly reduced such that 10-fold more *xnr2* mRNA is required to produce the same effect (compare 1 pg *xnr2* alone with 10 pg *xnr2* co-injected with 3 ng CM-*der* in Fig. 7A).

We used animal cap assays to understand the inhibitory activity of CM-Der on Nodal signaling. Embryos were injected with increasing amounts of *xnr2* mRNA either alone or in combination with 2 ng of CM-*der*. When animal cap explants were analyzed at stage 11, *xnr2* mRNA was found to induce *xbra* expression at both 1 and 10 pg. Consistent with the ability of Nodal ability to induce both dorsal and ventral mesoderm in a dose-dependent manner, *gsc* and *chordin* expression (both markers of dorsal mesoderm) were detected at 10 pg, but not at lower doses (Fig. 7B, lanes 1-3). Levels of *xnr2* that induced mesoderm formation also activated transcription of other TGFβs including *derrière* and *xnr1*. In the presence of CM-*der*, a ten-fold higher dose of *xnr2* mRNA was required for

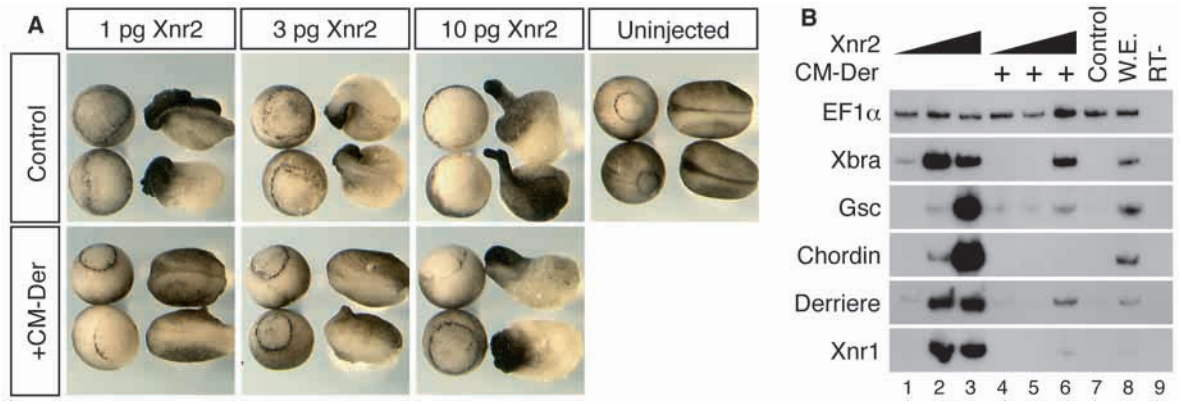

Fig. 7. CM-Der can antagonize Nodal-mediated mesoderm induction. (A) In whole embryos, CM-*der* attenuates *xnr2* overexpression phenotypes. One-cell embryos were injected at the animal pole with increasing doses of *xnr2* mRNA (top row; *xnr2* levels range from 1 to 10 pg). The overexpression phenotype is shown at stage 11 (left side of each panel) and stage 20 (right side of each panel). This overexpression phenotype is markedly decreased by co-injection of 3 ng of CM-*der* mRNA (bottom row). (B) In animal caps, CM-*der* antagonizes both *xnr2*-mediated mesoderm induction and the induction of other TGFβ transcripts. Embryos were injected at the animal pole at the one-cell stage with increasing amounts of *xnr2* mRNA (0.1, 1 and 10 pg) either alone or with 2 ng of CM-*der* mRNA. Co-injection of CM-*der* attenuates mesoderm induction by *xnr2* (as indicated by *xbra*, *gsc* and *chordin*) and also retards the induction of multiple TGFβ transcripts.

mesoderm induction, consistent with data from whole embryos (Fig. 7B, lanes 4-6). While 10 ng of *xnr2* mRNA was sufficient to induce extreme dorsal types of mesoderm on its own, when used in combination with CM-*der* only the general mesoderm marker *xbra* was seen. In addition, activation of other TGFβ transcripts was severely reduced, suggesting that the ability of CM-Der to inhibit Nodal signaling is at least partially due to antagonism of Nodal-induced *derrière*.

Derrière forms heterodimers with multiple members of the TGFβ superfamily

The observation that CM-Der inhibited a broad range of Xnr2 induced transcripts suggested to us that it may antagonize Nodal signaling directly through the formation of heterodimers. This possibility is suggested by the observation that BMPs readily form heterodimeric ligands within their subfamily (Aono et al., 1995; Hazama et al., 1995) and the recent discovery that Nodal proteins and BMPs might mutually antagonize one another through the formation of heterodimers (Yeo and Whitman, 2001). In addition, a cleavage mutant form of *xnr5* has been shown to block the mesoderm inducing activity of multiple TGFβ family members, including *derrière*, in animal caps (Onuma et al., 2002). We used an in vivo translation assay in *Xenopus* oocytes to study direct interactions between CM-Der and other members of the TGFβ family. Mature Nodal ligands have proven difficult to detect in either lysates or conditioned media from expressing cells, probably because of inefficient proteolytic processing, instability of the mature ligand or a combination of both factors (Constam and Robertson, 1999). We encountered similar problems with Derrière expression in *Xenopus* oocytes (not shown), but overcame it by employing HA-epitope tagged Activin/Nodal and Activin/Derrière chimeric proteins. As shown in Fig. 8 (supernatant αHA lanes 1 and 2), when the pro domains of Xnr2 and Derrière were replaced with that of

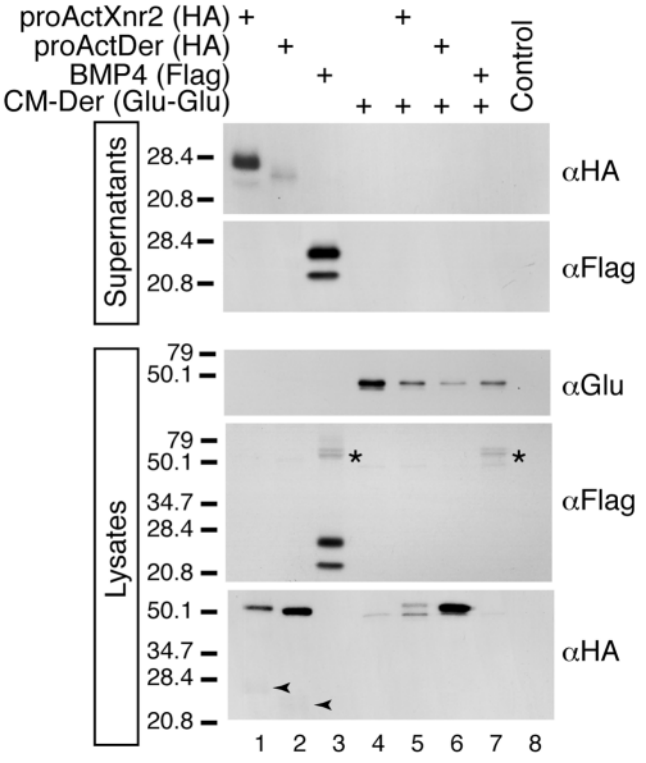

Fig. 8. CM-Der inhibits secretion of multiple members of the TGFβ superfamily. Oocytes were injected with synthetic mRNA encoding epitope tagged proAct-Xnr2 (HA tagged), proAct-Derrière (Flag tagged) or BMP4 either alone or in combination with CM-Der. Injected oocytes were cultured in the presence of [^{35}S]methionine and the supernatants and lysates analyzed by immunoprecipitation. Supernatants from oocytes expressing proAct-Xnr2, proAct-Derrière or BMP4 alone contain bands corresponding to the predicted sizes of the mature ligands, while the lysates also contain higher molecular weight unprocessed precursor proteins (lanes 1-3). CM-Der (tagged with the Glu-Glu epitope) is seen only in oocyte lysates and in its presence no mature Xnr2, Derrière or BMP4 ligands are detected in the supernatants (lanes 4-7). Unprocessed precursor proteins are still detected in oocyte lysates. Asterisks indicate unprocessed BMP4; arrowheads indicate mature Xnr2 and Derrière ligands.

Activin, detectable levels of the mature ligands were immunoprecipitated from oocyte supernatants. Co-injection of an equal amount of CM-*der* mRNA completely blocked secretion of Xnr2 and Derrière ligands from oocytes (Fig. 8, supernatant αHA lanes 5 and 6), demonstrating that the Derrière cleavage mutant can inhibit secretion of multiple proteins in the TGFβ family. We also found that secretion of Flag-tagged BMP4 was completely inhibited by CM-Der (Fig. 8, supernatant αFlag lanes 3 and 7). To confirm that all mRNAs were being translated in the oocytes, we carried out immunoprecipitations from lysates. All oocytes injected with Glu-Glu-tagged CM-*der* mRNA expressed an unprocessed protein of ~50 kDa (Fig. 8, lysate αGlu lanes 4-7). Unprocessed HA-tagged proAct-Derrière and proAct-Xnr2 were also immunoprecipitated from oocyte lysates, even when CM-Der was co-expressed (Fig. 8, lysate αHA lanes 1-2 and 5-6), indicating that inhibition occurs at the level of protein processing and secretion rather than mRNA translation. Very low levels of mature Derrière and Xnr2 ligands were detected only in lysates from oocytes not expressing CM-Der (mature ligands indicated by arrowheads). Similarly, unprocessed BMP4 was present in all oocyte lysates expressing Flag-tagged BMP4, but the mature ligand was lost upon co-expression of CM-Der (Fig. 8, lysate αFlag lanes 3 and 7; unprocessed protein bands are indicated with an asterisk).

To confirm that CM-Der is capable of inhibiting BMP activity, we analyzed its activity in animal cap explants. Untreated ectodermal explants develop in culture into undifferentiated epidermis; however, when BMP signaling is blocked by treatment with BMP-specific antagonists (Lamb et al., 1993; Sasai et al., 1995; Piccolo et al., 1996; Zimmerman et al., 1996), by use of dominant negative BMP ligands (Hawley et al., 1995) or through dissociation of the animal cap (Wilson and Hemmati-Brivanlou, 1995), neural fates are induced (for a review, see Harland, 2000). We reasoned that if CM-Der antagonizes members of the BMP subfamily, it should also be a direct inducer of neural tissue in animal caps. Stage 20 animal caps injected with 2 ng of CM-*der* mRNA were found to express the general neural markers *n-cam* and *nrp-1*, even in the absence of the mesodermal marker *muscle actin* (Fig. 9, lane 2). This induction occurred at the expense of the epidermal marker *epidermal keratin* (Fig. 9, lanes 2 and 3). The activity of CM-*der* was indistinguishable from that of the secreted BMP antagonist *noggin* (Fig. 9, lane 1).

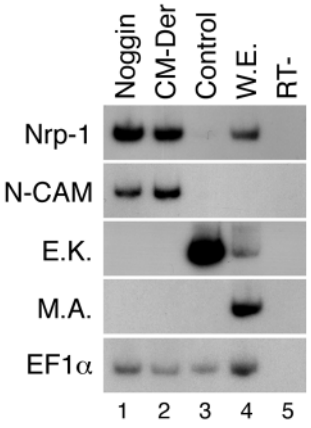

Fig. 9. CM-*der* induces neural tissue in ectodermal explants. Embryos were injected at the animal pole at the one-cell stage with 25 pg *noggin* mRNA or 2 ng CM-*der* mRNA and explants were cultured until stage 20 and analyzed by RT-PCR. Both *noggin*- and CM-*der*-treated animal caps express the neural markers *nrp-1* and *n-cam* in the absence of *muscle actin* (MA, a mesoderm-specific transcript) and at the expense of *epidermal keratin* (EK, an epidermal marker).

The most likely mechanism by which CM-Der could inhibit processing and secretion of Xnr2 and BMP4 is through heterodimerization. Multiple strategies were used to determine whether mature Derrière/Nodal and Derrière/BMP heterodimers formed in the *Xenopus* oocyte expression system. In the first, HA-tagged proAct-Derrière was co-expressed in oocytes with either Flag-tagged proAct-Xnr2 or untagged proAct-Xnr2. HA-tagged Derrière was then immunoprecipitated from the oocyte culture media using αHA antibody and resolved by SDS-PAGE. When expressed by itself, the mature Derrière ligand was present as a smear rather than a single band, migrating between 22 and 28 kDa (Fig. 10A, lane 3). This is most likely to be due to post-translational modifications such as glycosylation. When Flag-tagged proAct-Xnr2 was co-expressed with HA-tagged proAct-Derrière, αHA immunoprecipitation pulled down two distinct protein bands (Fig. 10A, lane 4). The first band in this doublet migrated from 19 to 23 kDa, while the second was a tighter band centered at 25 kDa. When Flag-tagged proAct-Xnr2 was replaced with untagged proAct-Xnr2, the mobility of the upper band shifted by slightly over 1 kDa (the expected size of the Flag epitope), while the lower band remained unchanged. Based on these data, we conclude that the upper band in the doublet is Xnr2 co-precipitating with the HA-tagged Derrière ligand. The equivalent intensities of the lower Derrière band and the upper Xnr2 band indicate that Derrière forms heterodimers with Nodal at least as efficiently as it forms homodimers with itself (Fig. 10A). The differences in mobility observed between Derrière alone and in combination with Xnr2 raise the possibility that heterodimerization affects glycosylation or other post-translational modifications.

The second method used to detect a direct association between the mature Derrière ligand and other members of the TGFβ family was a two-step immunoprecipitation relying on the disulfide-mediated covalent association between subunits. As before, HA-tagged proAct-Derrière was co-expressed in oocytes with various Flag-tagged TGFβs. HA-Derrière was than immunoprecipitated from supernatants using αHA antibody conjugated to protein A sepharose beads and bound proteins were released by boiling. Co-precipitating Flag-tagged proteins were then pulled down in a second immunoprecipitation, this time using αFlag antibody. Single-step immunoprecipitations using αHA or αFlag antibody alone confirmed that all mRNAs were efficiently translated by the oocytes (Fig. 10B, αFlag and αHA panels). Flag-tagged Derrière, Xnr2 and BMP4 were all detected after the two-step immunoprecipitation, demonstrating that Derrière is capable of heterodimerizing with BMPs and Nodal proteins in addition to forming homodimers (Fig. 10B, αHA:αFlag panel, lanes 5-7).

Finally, we used epitope tagged versions of wild-type Derrière and Xnr2 (rather than Activin/Derrière and Activin/Xnr2 fusion constructs) to examine heterodimerization between full-length, uncleaved proteins. This was done to eliminate the possibility that the activin pro domain was responsible for mediating heterodimerization not normally observed with wild-type ligands. This is not an idle concern, as Gray and Mason (Gray and Mason, 1990) have demonstrated that the pro domains of TGFβ ligands support dimerization. As noted previously, we were unable to detect significant levels of the mature Xnr2 and Derrière ligands in supernatants from oocytes expressing wild-type proteins.

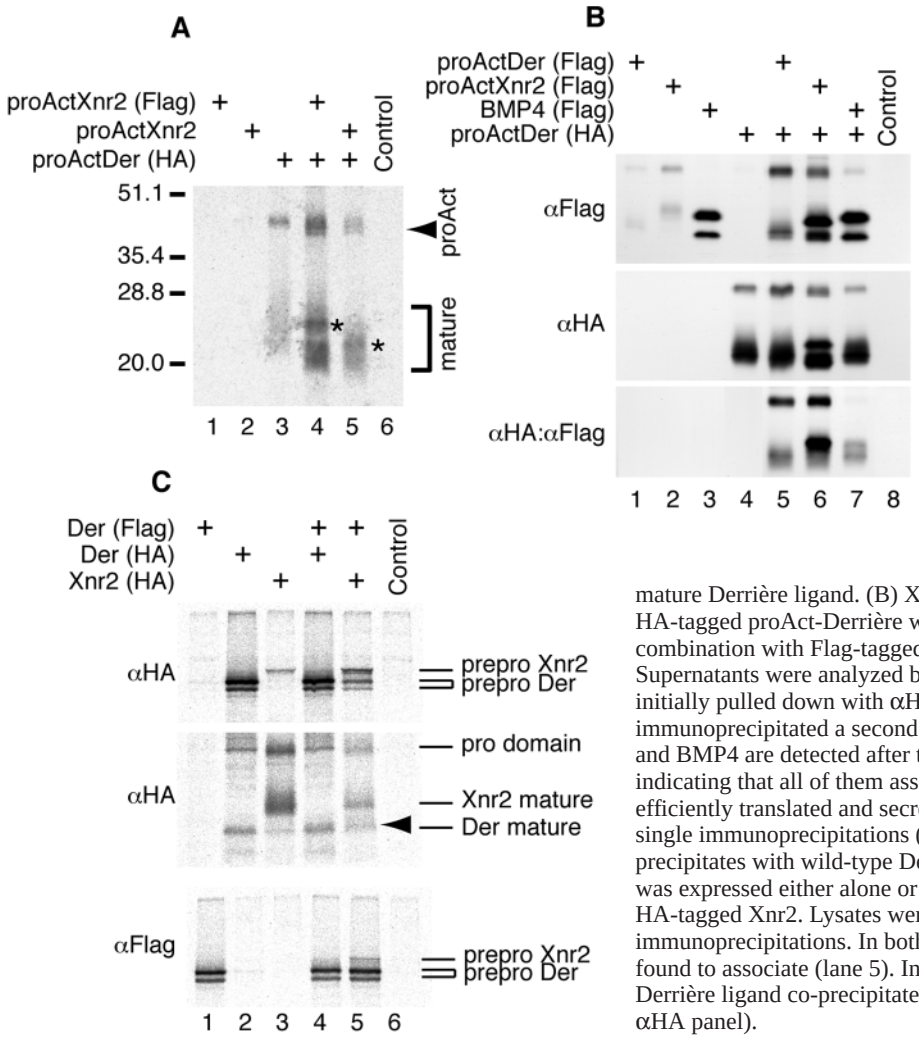

Fig. 10. Derrière physically associates with other members of the TGFβ superfamily, including Xnr2 and BMP4. (A) HA-tagged proAct-Derrière was co-expressed in *Xenopus* oocytes along with either Flag-tagged proAct-Xnr2 or untagged proAct-Xnr2. When HA-tagged proAct-Derrière is expressed on its own and immunoprecipitated with αHA, the mature ligand is seen as a broad range band between 22 and 28 kDa (lane 3). In the presence of Flag-tagged proAct-Xnr2, two distinct bands are apparent (lane 4); the removal of the Flag epitope tag from proAct-Xnr2 (a decrease in molecular weight of approximately 1 kDa) causes a corresponding mobility shift in the upper of the two bands (mature Xnr2 bands marked by asterisks). Note that co-expression of Derrière and Xnr2 also causes an overall decrease in the molecular weight of the mature Derrière ligand. (B) Xnr2 and BMP4 co-precipitate with Derrière. HA-tagged proAct-Derrière was expressed in oocytes either alone or in combination with Flag-tagged proAct-Derrière, proAct-Xnr2 or BMP4. Supernatants were analyzed by two-step immunoprecipitation; proteins were initially pulled down with αHA coupled to protein A, released by boiling and immunoprecipitated a second time using αFlag. Flag-tagged Derrière, Xnr2 and BMP4 are detected after the double immunoprecipitation (lanes 5-7), indicating that all of them associate with HA-tagged Derrière. All proteins are efficiently translated and secreted into the supernatant, as demonstrated by single immunoprecipitations (top two panels). (C) Wild-type Xnr2 co-precipitates with wild-type Derrière in oocyte lysates. Flag-tagged Derrière was expressed either alone or in combination with HA-tagged Derrière or HA-tagged Xnr2. Lysates were analyzed by αHA and αFlag immunoprecipitations. In both cases, unprocessed Xnr2 and Derrière were found to associate (lane 5). In addition, low levels of the mature Flag-tagged Derrière ligand co-precipitated with HA-tagged Xnr2 (arrowhead, bottom αHA panel).

However, the full-length, uncleaved proteins could be readily extracted and immunoprecipitated from oocyte lysates. Unprocessed (prepro) Derrière is present in the oocyte lysate as a doublet, while prepro Xnr2 is present as a single band; these bands migrate at distinct molecular weights and can be resolved by SDS-PAGE (Fig. 10C, lanes 1-3). When Flag-tagged Derrière and HA-tagged Xnr2 were co-expressed in oocytes and immunoprecipitated using αHA, the prepro Derrière doublet was clearly seen to co-precipitate with prepro Xnr2 (Fig. 10C, lane 5, upper αHA panel). When the reciprocal immunoprecipitation was carried out using αFlag, prepro Xnr2 was pulled down with prepro Derrière (Fig. 10C, lane 5, αFlag panel). In addition, mature Derrière and Xnr2 ligands were detected in oocyte lysates after αHA immunoprecipitation (Fig. 10C, lanes 1-3, middle αHA panel). When HA-tagged Xnr2 and Flag-tagged Derrière were co-expressed and pulled down using αHA, a band migrating at the expected size of the mature Derrière ligand was seen to co-precipitate (Fig. 10C, lane 5, arrowhead). These data, coupled with our observation that CM-Derrière (possessing the wild-type Derrière pro domain) efficiently inhibits the processing and secretion of wild-type BMP4, proAct-Derrière and proAct-Xnr2 (Fig. 9), provide convincing evidence that the naturally occurring forms of diverse TGFβ family members efficiently heterodimerize in co-expressing cells.

DISCUSSION

Derrière and members of the Nodal family act cooperatively in *Xenopus* mesoderm induction

Members of the Nodal family have recently emerged as essential components of mesoderm induction in species ranging from frog to mouse. Establishing the full importance of Nodal signaling has been complicated by the fact that there are at least five different Nodal-like proteins expressed in early *Xenopus* embryogenesis capable of inducing mesoderm. All are expressed in the vegetal endoderm and the marginal zone beginning shortly after MBT, with considerably higher levels detected on the dorsal side of the embryo (Jones et al., 1995; Joseph and Melton, 1997; Takahashi et al., 2000). The ability of the Nodal-specific antagonist Cer-S to completely block mesoderm formation in *Xenopus* has led to the suggestion that a dorsal to ventral gradient of Nodal signaling may be responsible for mediating mesoderm induction and patterning in response to VegT and β-catenin (Agius et al., 2000). While

not contradicting this basic model, we find that other members of the TGFβ superfamily, particularly Derrière, are also likely to play a significant role in this process.

While Derrière has previously been implicated in anteroposterior patterning of the mesoderm during gastrulation (Sun et al., 1999), we suggest that its expression in late blastula stages is indicative of a role in general mesoderm induction in response to VegT activity. More than any of the Nodal family members, *derrière* transcripts mirror the expression pattern of zygotic *vegT* and known targets of VegT signaling such as *bix4* (Fig. 1). Like the mesodermal marker *xbra*, *derrière* is expressed in both the superficial and deep cells of the marginal zone at stage 10+ and transcript levels are more or less equally distributed between dorsal and ventral sides of the embryo. *derrière* expression is also detected in cells of the sub-blastoporal endoderm, which are able to mediate mesoderm induction. Finally, we find that *nodal* activity sufficient to induce mesoderm also invariably activates *derrière* transcription and the Nodal-specific antagonist *cer-S* blocks *derrière* expression in the whole embryo (Fig. 3).

Taken together, these data strongly suggest that mesoderm induction is a cooperative process in *Xenopus* that involves VegT and multiple members of the TGFβ superfamily. Derrière and members of the Nodal family are each capable of inducing one another in animal cap assays (Fig. 3L, Fig. 7B) (Takahashi et al., 2000; Onuma et al., 2002), creating a positive transcriptional feedback loop that is probably mediated in part through the maternal transcription factor FAST-1 (Osada et al., 2000). In addition, each of these ligands can induce Smad2 phosphorylation (an essential step in the transduction of Activin-like mesoderm-inducing signals) and expression of zygotic *vegT* (Lee et al., 2001). The probable importance of this feedback loop for mesoderm formation is demonstrated by the observation that the lowest level of Xnr2 capable of generating mesoderm is also the lowest level that induces transcription of *derrière* and other *nodal* family members (Fig. 7B, lanes 2 and 6). The same is found to be true for mesoderm induction by *derrière* (not shown). Thus, these TGFβ family members act cooperatively to induce and maintain their mutual expression.

It is also interesting to note that Derrière, unlike Nodal proteins and Activin, does not appear to strongly favor the formation of dorsal over ventral mesoderm even at relatively high doses (Fig. 2). This difference may be due in part to the late induction of Smad2 phosphorylation by Derrière in comparison to Xnrs (Lee et al., 2001). It has been hypothesized that differences in the timing of Smad2 activation may explain how different ligands are able to produce different activities while using the same intracellular pathway (Lee et al., 2001). In *Xenopus* embryogenesis, Smad2 phosphorylation is initiated dorsally at stage 9 but is essentially equivalent in dorsal and ventral halves of the embryo by the onset of gastrulation (Faure et al., 2000; Lee et al., 2001). This suggests that early dorsal Smad2 phosphorylation arises from Xnr activity, while later Smad2 phosphorylation may rely on Derrière, which is expressed more uniformly along the dorsoventral axis than Xnrs. In this model, early activation of Smad2 leads to organizer fates, while later activation is compatible ventral and lateral fates in addition to dorsal ones.

Ultimately, our observation that induction of dorsal and ventral markers by Derrière is dependent on the context of the embryo emphasizes the importance of the dorsoventral prepattern imposed by β-catenin accumulation (Heasman et al., 1994; Schneider et al., 1996; Rowning et al., 1997). The presence of this dorsal modifier overrides the late activation of Smad2 by Derrière to induce organizer-specific transcripts, while its absence causes ventrolateral gene expression. Thus, while timing of Smad2 activation may contribute to dorsoventral patterning (Lee et al., 2001), early Smad signaling is not necessary for organizer formation in the context of β-catenin activity. Mechanistically, the two pathways are known to converge and synergize at *cis*-regulatory sequences to induce organizer-specific genes (Watabe et al., 1995). Even elevated levels of Nodal expression on the dorsal side, which may impart specific dorsoventral patterning information, can be attributed to transcriptional activation by β-catenin (Agius et al., 2000) and repression by ventral inducers such as BMP4 (Osada et al., 2000).

Diverse members of the TGFβ superfamily readily form heterodimers

Interaction between Nodal proteins and other members of the TGFβ family can occur at the level of ligand heterodimerization as well as transcriptional activation. Here, we have shown in immunoprecipitation assays that the mature Derrière ligand physically associates with Nodal proteins and BMPs (Fig. 10A,B). In addition, unprocessed Derrière and Nodal heterodimers are found in the lysate of co-expressing oocytes (Fig. 10C). Consistent with this finding, CM-Der is capable of blocking expression of processed Xnr2 and BMP4 protein and directly neuralizing ectodermal explants (Figs 8, 9). This extends the findings of Yeo and Whitman (Yeo and Whitman, 2001), who have shown that heterodimers can form between cleavage mutant Nodal and BMPs. While our experiments cannot definitively address whether heterodimerization occurs at physiological levels in the embryo, the efficient interaction between Flag-tagged Xnr2 and HA-tagged Derrière in co-expressing oocytes suggest that heterodimers would form. This conclusion is reinforced by the fact that secretion of Xnr2 from oocytes can be efficiently blocked by co-injection of an equivalent amount of CM-*der* mRNA (Fig. 8).

It is interesting to speculate on how and to what degree the activity of heterodimers may differ from that of homodimers. There is compelling evidence from studies of the BMP subfamily that heterodimerization has the potential to modulate activity of TGFβ ligands. BMP2/7 and BMP4/7 heterodimers appear to form preferentially over homodimers and display increased activity in assays for osteogenic differentiation (Aono et al., 1995; Hazama et al., 1995) and mesoderm induction (Suzuki et al., 1997; Nishimatsu and Thomsen, 1998). A complete evaluation of the activity of Derrière/Nodal and Derrière/BMP heterodimers is beyond the scope of the present study; however, the altered electrophoretic mobility of processed Xnr2 and Derrière (Fig. 10A,B) when expressed together strongly suggests differences in post-translational modification. What importance, if any, these differences have for endogenous signaling remains to be determined.

Concerns pertaining to the use of TGFβ cleavage mutants as dominant negative reagents

A wide variety of dominant-negative strategies have been

developed for carrying out loss-of-function studies in *Xenopus* because genetic techniques for removing zygotic gene function are not readily available (Lagna and Hemmati-Brivanlou, 1998). In the TGFβ superfamily, proteolytic cleavage mutants have proven especially useful for this task (Lopez et al., 1992; Wittbrodt and Rosa, 1994; Hawley et al., 1995; Joseph and Melton, 1998). All TGFβs are synthesized as an inactive precursor protein in which the pro and mature domains are separated by a proteolytic cleavage site with an RXXR motif. Alteration of this motif inhibits proteolytic processing and heterodimerization between wild-type and mutant monomers in co-expressing cells blocks the release of active dimers. A high degree of specificity has been claimed for these dominant-negative reagents. In this study we report several findings that raise concerns about both the specificity of cleavage mutants and the degree to which inhibition of proteolytic processing in the Nodal family blocks signaling.

Our analysis of the discrepancies between Nodal loss-of-function studies using CM-Xnr2 (Osada and Wright, 1999) and those using Cer-S (Agius et al., 2000) revealed that cleavage mutant forms of Nodal retain some signaling capacity. These data suggest that formation of mesoderm in *Xenopus* embryos injected with CM-Xnr2 can be explained by incomplete inhibition of Nodal signaling. Such an explanation fits with the phenotypes reported for CM-Xnr2-expressing embryos. Defects include a delay in the formation of the blastopore lip during gastrula stages and a variety of anterior defects, such as microcephaly, anencephaly and shortened body axes, later in development. All of these may be secondary consequences of disorganized mesoderm formation or partial inhibition of involution at gastrulation because of attenuated Nodal activity. The observation that CM-Xnr2 blocked elongation in response to wild-type Nodal proteins is consistent with our findings that the activity of CM-Xnr2 is severely attenuated and, as a result, it fails to induce dorsal mesodermal fates. As dorsal mesoderm undergoes the most extreme convergent extension, and CM-Xnr2 can heterodimerize with wild-type Nodal proteins and block their ability to induce dorsal fates (data not shown), the failure of animal caps to elongate probably reflects a conversion to ventral and lateral mesoderm fates. In addition, cleavage mutant forms of both Xnr1 and 4 were found to possess mesoderm-inducing activities comparable with unaltered ligands, suggesting this may be a general property of the Nodal family (Osada and Wright, 1999).

While the presence of putative proteolytic cleavage sites N-terminal to the canonical cleavage site in Xnr1, 2 and 4 may partially explain the residual activity of cleavage mutants, it cannot be the sole factor at work. A mutant that lacks both RXXR motifs had activity comparable with the single mutant (Fig. 4B). Even completely replacing the Xnr2 pro-domain with that of Activin (which has been shown to be cleaved at a single site in oocyte expression studies) (Hawley et al., 1995) failed to produce an authentic dominant-negative Nodal construct. These data indicate that unprocessed Nodal protein is capable of generating an attenuated Nodal-like signal. A similar activity has been reported for the TGFβ family member Lefty A, which can activate the MAPK pathway as an unprocessed precursor (Ulloa et al., 2001). However, the ability for unprocessed TGFβ proteins to transduce a wild-type signal appears to be the exception rather than the rule. In the same battery of assays (including whole embryos, animal caps, and in vivo translation and secretion),we confirmed that the Derrière cleavage mutant was devoid of all activity. Other studies using cleavage mutant forms of Activin, TGFβ, Vg1, BMPs and Xnr5 and Xnr6 all reach the same conclusion (Lopez et al., 1992; Wittbrodt and Rosa, 1994; Hawley et al., 1995; Joseph and Melton, 1998; Onuma et al., 2002). Consequently, the use of cleavage mutants for conducting loss-of-function studies can be valid, provided the effects of heterodimerization are taken into account.

A second, and potentially more serious, concern with the use of cleavage mutant proteins as dominant negative reagents relates to their specificity. The TGFβ superfamily contains many members and several distinct subfamilies. For a dominant negative reagent to be useful, its inhibitory activity should be as restricted as possible, preferably limited to the specific molecule in question or a few closely related family members. The promiscuous heterodimerization that we have observed between Derrière, Nodal and BMP ligands suggests that precise specificity may be impossible to achieve with cleavage mutants. These concerns are verified by our finding that CM-Der can induce neural fates in animal caps (Fig. 9), an activity that almost certainly arises from promiscuous heterodimerization with endogenous BMPs. At the very least, dominant-negative TGFβ constructs must be carefully evaluated for their effects on other family members, ideally using a combination of in vivo expression and embryonic activity assays.

In conclusion, we report that both the mesoderm-inducing effect of *derrière* and the promiscuous blocking activity of CM-*derrière* are consistent with the anteroposterior patterning activity of these reagents (Sun et al., 1999). *derrière* induces ventral or paraxial mesoderm, with its well-established posteriorizing activity (for a review, see Munoz-Sanjuan and Brivanlou, 2001), and therefore tends to suppress anterior structures. It is therefore an indirect posteriorizing factor as suggested by Sun et al. (Sun et al., 1999). By contrast, CM-*derrière* blocks the formation of mesoderm, and by blocking BMP activity also induces neural structures, a combination that results in anterior fates (reviewed by Harland, 2000). In more general terms, our findings suggest that interactions between different members of the TGFβ superfamily can be highly complex, involving reciprocal activation at the transcriptional level and direct physical association through the formation of heterodimers. While these multiple levels of crosstalk make it impossible to separate completely the requirement for individual TGFβs in the process of mesoderm formation in *Xenopus*, the preponderance of evidence points towards a model of cooperative induction in which both Nodal proteins and Derrière are key participants.

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