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The TGF-β family member *derrière* is involved in regulation of the establishment of left–right asymmetry

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Although a number of genes that are involved in the establishment of left–right asymmetry have been identified, earlier events in the molecular pathway developing left–right asymmetry remain to be elucidated. Here we present evidence suggesting that the transforming growth factor-β family member *derrière* is involved in the development of left–right asymmetry in *Xenopus* embryos. Ectopic expression of *derrière* on the right side can fully invert cardiac and visceral left–right orientation and *nodal* expression, and expression of a dominant-negative form of *derrière* on the left side can partially randomize the left–right orientation and *nodal* expression. Moreover, while expression of the dominant-negative *derrière* does not inhibit the activity of Vg1 directly, it can rescue the altered left–right orientation induced by Vg1. Vg1 can induce *derrière* in animal cap explants. These results suggest that *derrière* is involved in earlier molecular pathways developing the left–right asymmetry.

INTRODUCTION

In the animal body plan, the left–right axis is specified in accord with the development of anterior–posterior and dorsal–ventral axes (Brown and Wolpert, 1990). Recently, a number of molecules that are involved in the promotion and establishment of left–right asymmetry have been identified (Harvey, 1998; Ramsdell and Yost, 1998; Beddington and Robertson, 1999; Capdevila *et al.*, 2000). Of such molecules, members of the transforming growth factor-β (TGF-β) superfamily are conserved factors implicated in the pathway establishing left–right asymmetry. The expression of *nodal* and *lefty2* in the left lateral plate mesoderm (LPM) is strictly correlated with the development of normal organ *situs* (Harvey, 1998; Ramsdell and Yost, 1998; Beddington and Robertson, 1999). *nodal* is antagonized by *lefty2* (Meno *et al.*, 1999) and induces *Pitx2*, a transcription factor that directs subsequent morphological decisions (Harvey, 1998; Ramsdell and Yost, 1998; Beddington and Robertson, 1999). Misexpression of upstream laterality genes disrupts the normal expression pattern of *nodal* and *lefty2*, and randomizes heart looping (Lohr *et al.*, 1997; Sampath *et al.*, 1997; Meno *et al.*, 1998; Pagan-Westphal and Tabin, 1998).

Although progress has been made in understanding the molecular mechanism underlying vertebrate left–right patterning, very little is known about the processes that are involved in first developing left–right asymmmetry in the embryo. Recent studies using mice lacking the kinesin superfamily member KIF3A or KIF3B suggest that rotation of nodal cilia is required for the initial breaking of symmetry at the node (Nonaka *et al.*, 1998; Okada *et al.*, 1999; Takeda *et al.*, 1999), although whether this mechanism is universal or not is unknown. Studies in chick and *Xenopus* suggest that the left–right axis is initiated prior to gastrulation. In the chick, left–right asymmetry of the node is inductively patterned by signals from lateral tissues. Once induced for left–right identity, the node then directs left–right development in adjacent tissues (Pagan-Westphal and Tabin, 1998). In *Xenopus*, it has been suggested that the activity that confers left–right identity to the organizer/node is mediated by Vg1, a member of the TGF-β superfamily. One of the key observations was that while various experimental

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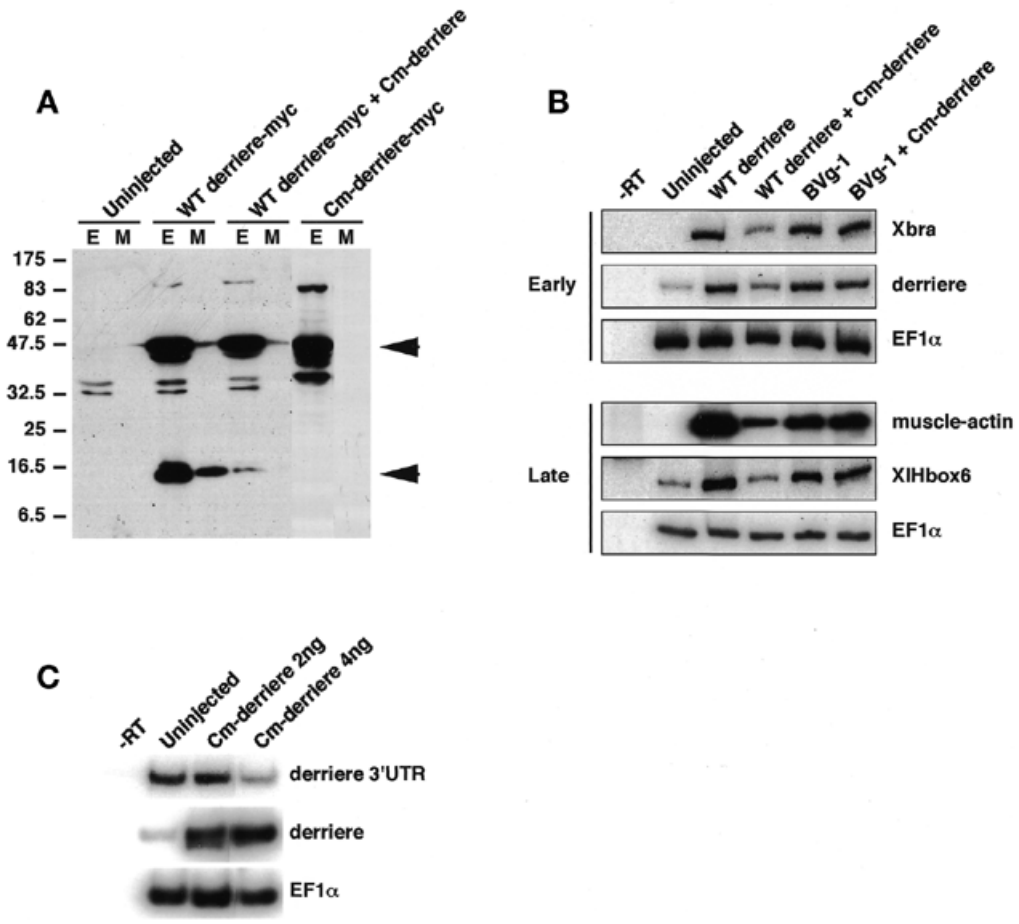

Fig. 1. Detection of a processed, mature form of wild-type *derrière* and the dominant-negative effect of a cleavage-deficient mutant of *derrière* (Cm-*derrière*). (**A**) Detection of a secreted mature form of *derrière* in both the whole-embryo extracts (E) and the conditioned media (M) from the wild-type (WT) *derrière* (WT *derrière*-Myc, 100 pg) injected embryos, but not in those from the Cm-*derrière* (a cleavage-deficient mutant of *derrière*) (Cm-*derrière*-Myc, 100 pg) injected embryos. Co-injection of Cm-*derrière* (non-Myc-tagged, 1 ng) with WT *derrière* (WT *derrière*-Myc, 100 pg) caused a marked decrease in the mature form. Arrowheads show an immature proprotein (upper) and a mature form (lower) of *derrière*. (**B**) A secreted *derrière* can induce expression of mesodermal marker genes in animal caps, like mature Vg1, and Cm-*derrière* can inhibit WT *derrière* specifically. Animal caps were dissected at blastula stage and cultured in the conditioned media obtained from WT *derrière*-, WT *derrière* plus Cm-*derrière*-, BVg1- or BVg1 plus Cm-*derrière*-injected oocytes until sibling embryos reached stage 11 (early) or stage 26 (late). Expression of *Xbra*, *derrière*, *EF1*α, *muscle-actin* and *XlHbox6* was analyzed by RT–PCR. *EF1*α served as a loading control. No signal was observed in the absence of reverse transcription (–RT). (**C**) Expression of Cm-*derrière* reduced endogenous *derrière* expression. Expression of endogenous *derrière* mRNA was analyzed by RT–PCR using the primer pair designed from the 3′-untranslated region of *derrière* (*derrière* 3′UTR), and expression of endogenous plus exogenous *derrière* mRNA was analyzed by RT–PCR using the primer pair designed from the coding region of *derrière* (*derrière*). *EF1*α served as a loading control.

manipulations lead to randomization, only Vg1 can fully invert cardiac and visceral left–right orientation and expression of a downstream laterality gene, *nodal* (Hyatt *et al.*, 1996; Hyatt and Yost, 1998). Here we present evidence suggesting that *derrière*, a member of the TGF-β superfamily, is also involved in establishing left–right asymmetry. *derrière* is a recently identified Vg1-related factor and is required for normal mesodermal patterning, paticularly in posterior regions of the *Xenopus* embryo (Sun *et al.*, 1999). Our results show that ectopic expression of *derrière* on the right side can also fully invert cardiac and visceral left–right orientation and *nodal* expression. More importantly, expression of a dominant-negative form of *derrière* on the left side can randomize the left–right orientation. In *Xenopus*, the tissues lateral to the organizer that carry left–right identity and are capable of inducing left–right specification of the organizer have been termed the 'left–right coordinator' (LRC) (Hyatt and Yost, 1998). Our results suggest that, like Vg1, *derrière* may act as one of the LRC factors in the left lateral vegetal cells in *Xenopus* embryos.

RESULTS AND DISCUSSION

derrière proprotein is efficiently processed to a functional, secreted form in embryos

To find out whether *derrière* is processed to a mature form in embryos, we constructed a C-terminal Myc epitope-tagged *derrière* (*derrière*-Myc) and injected its mRNA into the animal

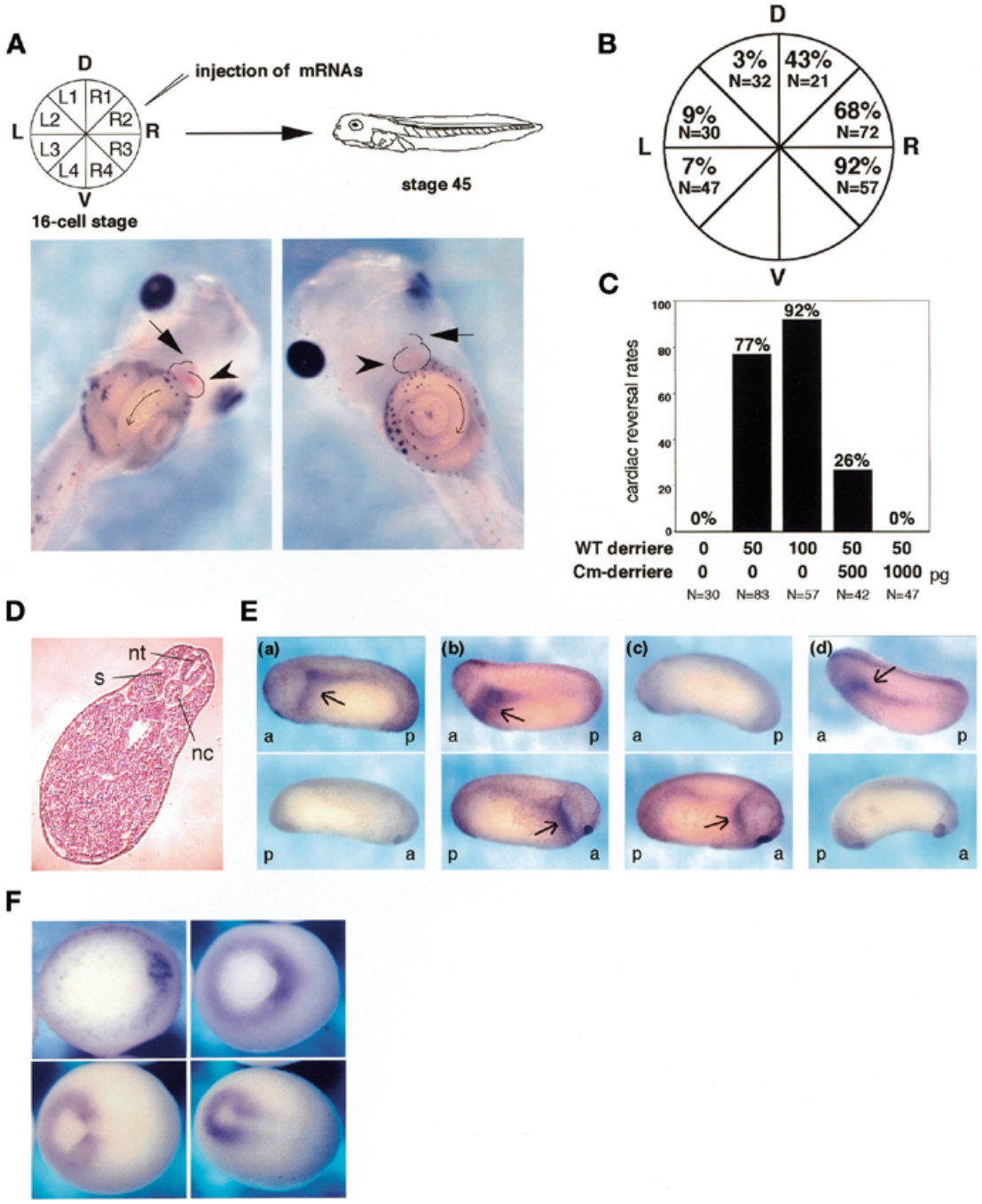

Fig. 2. Effect of *derrière* on left–right development and *Xnr-1* expression. (**A**) Experimental scheme (upper) and *situs solitus* (lower, left panel) and *situs inversus* (lower, right panel) phenotypes in *Xenopus*. Cell designations according to Hyatt and Yost (1998) are shown (upper, left). Embryos were injected at the indicated blastomere with mRNA at the 16-cell stage and were harvested at stage 45 for observation of cardiac and visceral left–right orientation. In *situs solitus* (left, control), embryos show normal heart orientation, with outflow tract (arrow) to the embryo's right and ventricle (arrowhead) to the left, and normal gut orientation with counterclockwise coiling. In *situs inversus* (right, *derrière*-injected into R3), embryos show reversed heart orientation, with outflow tract (arrow) to the embryo's left and ventricle (arrowhead) to the right, and reversed gut orientation with clockwise coiling. (**B**) Cardiac left–right reversal by injection of WT *derrière*. Embryos were injected with 100 pg of WT *derrière* mRNA in the indicated region. The percentages shown here indicate the cardiac reversal rates. (**C**) Cm-*derrière* can rescue the inversion of cardiac orientation induced by injection of WT *derrière* into R3. The indicated doses of mRNAs were injected into R3 at the 16-cell stage and the embryos were observed at stage 45 for cardiac and visceral left–right orientation. The percentages shown here indicate the cardiac reversal rates. (**D**) Transverse section of an embryo injected with WT *derrière* mRNA into R3. nt, neural tuge; nc, notochord; s, somite. (**E**) Altered *Xnr-1* expression patterns by injection of *derrière*. Embryos were collected at stages 22–24 and processed for whole-mount *in situ* hybridization analysis of *Xnr-1* expression. Upper panels, lateral view with anterior to the left (a, anterior; p, posterior); bottom panels, lateral view with anterior to the right. Embryos injected with 50 pg of WT *derrière* mRNA into L3, R2 and R3 exhibited normal left expression of *Xnr-1* (a), bilateral expression of *Xnr-1* (b) and right expression of *Xnr-1* (c), respectively. *Xnr-1* expression in an uninjected embryo is shown in (d). Arrows show *Xnr-1* staining. See the text for details. (**F**) Whole-mount *in situ* hybridization of *derrière*. All embryos are viewed from the vegetal surface with dorsal to the right. Upper left panel, stage 10.5; upper right panel, stage 11; bottom left panel, stage 12; bottom right panel, stage 13. Expression of *derrière* is symmetrical at all stages shown.

pole of 2-cell-stage embryos. Immunoblotting with anti-Myc antibody detected a processed, mature form in both the whole-embryo extracts and the conditioned media from the cultured embryos (Figure 1A, WT *derrière*-Myc, lower arrowhead). We then constructed a cleavage-deficient mutant of *derrière* (Cm-*derrière*-Myc) according to the design of the cleavage-deficient mutant of BMP (Hawley *et al.*, 1995). As expected, Cm-*derrière* was not processed to a mature form (Figure 1A, Cm-*derrière*-Myc). Moreover, co-injection of a 10-fold excess of Cm-*derrière* (non-Myc-tagged) with WT *derrière* (-Myc) significantly inhibited generation of the mature form (Figure 1A, WT *derrière*-Myc + Cm-*derrière*). To test the activity of the secreted, mature form of *derrière*, we collected conditioned media from defolliculated oocytes, which were injected with the indicated combinations of mRNAs shown in Figure 1B and incubated for 3 days. When tested in animal cap explants, a conditioned medium from WT *derrière*-injected oocytes induced expression of early and late mesodermal markers, like mature Vg1 (Figure 1B). A conditioned medium from oocytes co-injected with Cm-*derrière* and WT *derrière* had a significantly weaker ability to induce these mesodermal markers than that from the WT *derrière*-injected oocytes. In contrast, the ability of a conditioned medium from BVg1 (a chimeric BMP2-Vg1 protein, which is cleaved to the mature Vg1 protein) (Thomsen and Melton, 1993) injected oocytes to induce marker genes was unaffected by co-injection of Cm-*derrière* (Figure 1B).

Taken together, these results demonstrate that *derrière* proprotein is efficiently processed to a functional, secreted form in embryos, and that Cm-*derrière* functions as a specific dominant-negative construct and thus does not block Vg1. As WT *derrière* enhanced *derrière* mRNA expression (Figure 1B), it is possible that *derrière* expression is regulated by an autofeedback mechanism. To assess this possibility, we injected Cm-*derrière* mRNA into the marginal zone of four blastomeres at the 4-cell embryo stage and isolated total RNA at early gastrulation. To detect endogenous *derrière* transcripts, we used a primer pair designed from a 3′ untranslated region of *derrière* mRNA (Figure 1C, *derrière* 3′UTR). The RT–PCR analysis showed that expression of Cm-*derrière* reduced an endogenous *derrière* expression level (Figure 1C), suggesting the existence of a positive feedback mechanism of *derrière* expression.

Ectopic expression of *derrière* on the right side can invert cardiac and visceral left–right orientation

As the prospective mature region of *derrière* is ~80% identical to that of Vg1, we examined whether or not *derrière* also has the ability to affect the development of the left–right asymmetry. We injected *derrière* mRNA into various vegetal cells of the 16-cell-stage embryos and observed cardiac and visceral left–right orientation of the stage 45 embryos, as illustrated in Figure 2A. In the normal embryo, the ventricle is situated on the left side, with the outflow tract looping to the right side, and gut coils counterclockwise (Figure 2A, left). Because disruption of dorsoanterior or dorsal midline development is known to affect laterality (Danos and Yost, 1996; Lohr *et al.*, 1997; Nascone and Mercola, 1997), we scored only embryos with an apparently undisturbed primary axis, in which notochord, neural tube and somites appeared to be unaffected in injected embryos (Figure 2D). Injection of WT *derrière* into R3 resulted in the reversal of

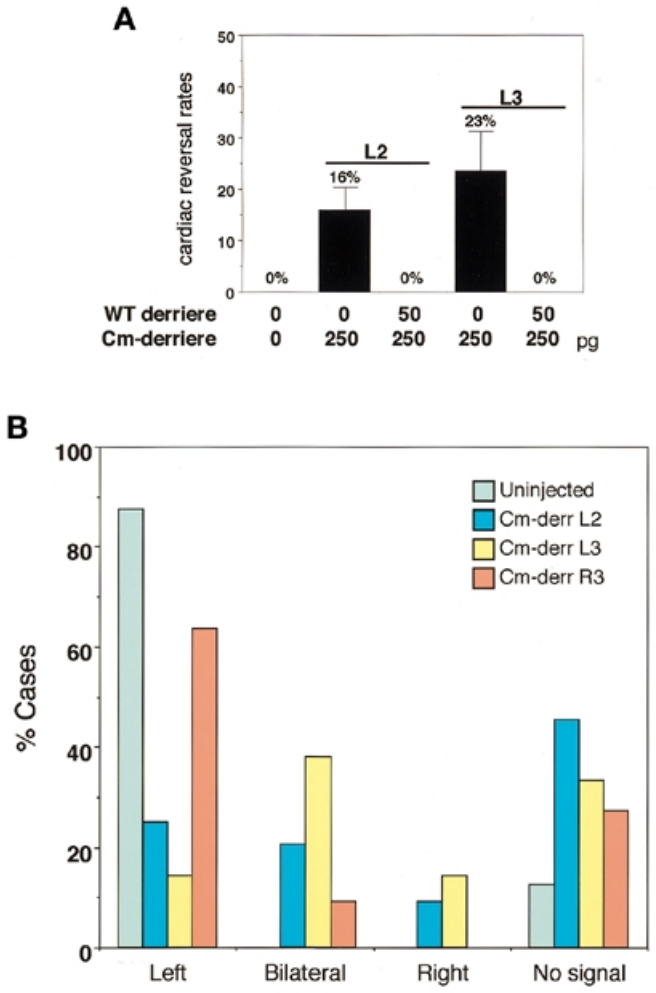

Fig. 3. Expression of Cm-*derrière* disturbs left–right asymmetry. (**A**) Embryos were injected with 250 pg of Cm-*derrière* mRNA or 250 pg of Cm-*derrière* mRNA plus 50 pg of WT *derrière* mRNA in L2 or L3 at 16-cell stage. Expression of Cm-*derrière* altered the orientation of cardiac looping (16% L2, SD = 4.6%, *N* = 120; 23% L3, SD = 7.8%, *N* = 118), and co-injection of WT *derrière* rescued completely the alteration of cardiac orientation induced by Cm-*derrière* (0% L2; *N* = 35; 0% L3; *N* = 42). (**B**) *Xnr-1* expression patterns in Cm-*derrière*-injected embryos. The percentages of cases of indicated *Xnr-1* expression in Cm-*derrière* L2 injected (blue, *N* = 44), Cm-*derrière* L3 injected (yellow, *N* = 42), Cm-*derrière* R3 injected (orange, *N* = 22) and uninjected (green, *N* = 32) embryos are shown.

the left–right orientation of cardiac looping and gut coiling in almost all the injected embryos (Figure 2A, right and 2B). The injection into R2 or R1 also induced inversion of the cardiac and visceral left–right orientation in about half of the population (Figure 2B), and the embryos often showed heterotaxia (not shown). In contrast, injection of WT *derrière* into L1, L2 or L3 had little or no effect on the left–right asymmetry (Figure 2B). The reversal effect of WT *derrière* expression in R3 on the left–right asymmetry was dose dependent and rescued completely by co-injection of a 20-fold excess of Cm-*derrière* (Figure 2C). Thus, Cm-*derrière* can in fact function dominant negatively.

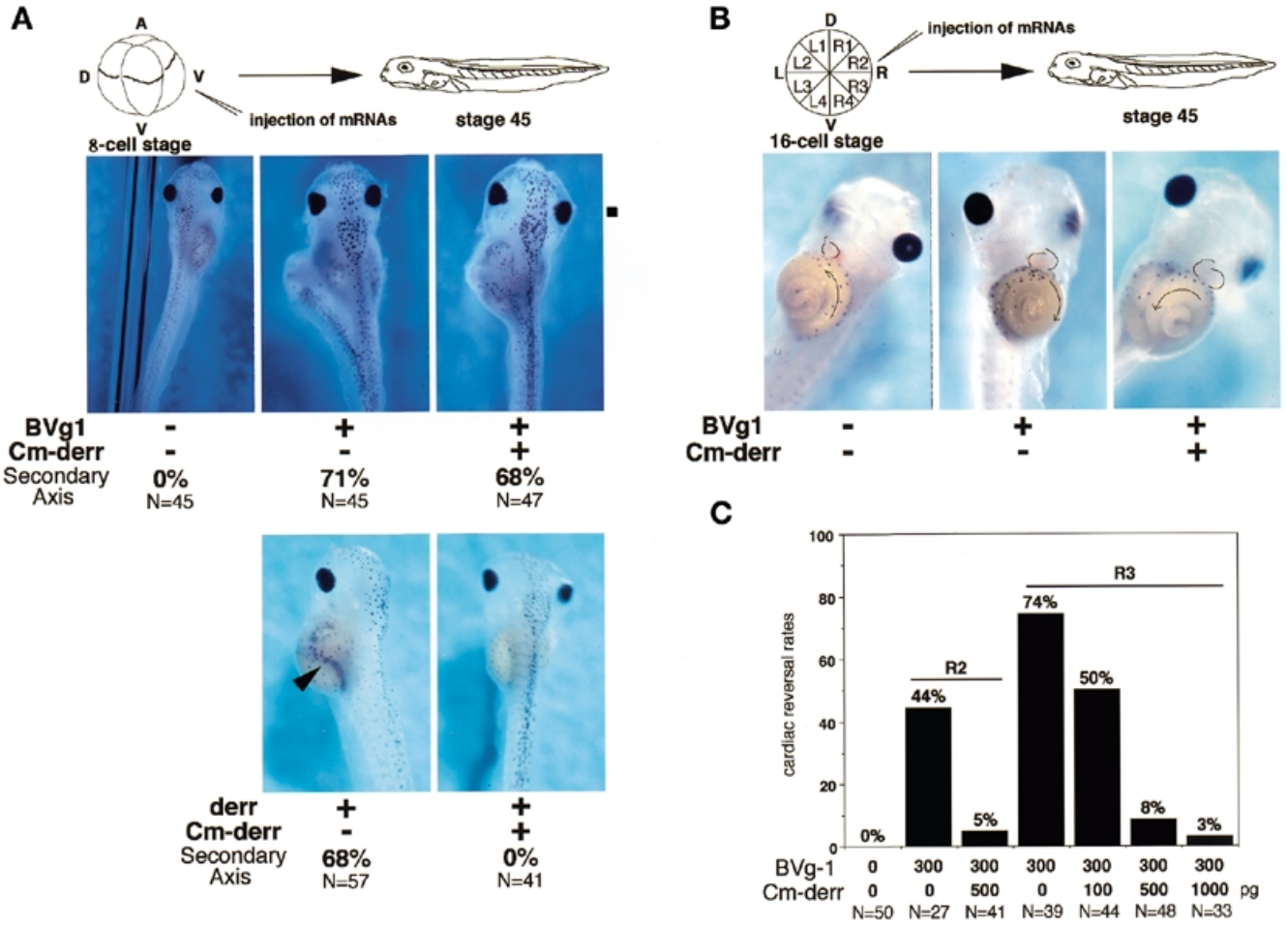

Fig. 4. Expression of Cm-*derrière* rescues the inversion of cardiac and visceral orientation induced by BVg1. (**A**) Effect of Cm-*derrière* on the ability of BVg1 to induce a secondary axis. Upper panels, embryos were injected with 300 pg of BVg1 mRNA or 300 pg of BVg1 mRNA plus 500 pg of Cm-*derrière* mRNA in the ventrovegetal blastomere at 8-cell stage and were harvested at stage 45 for morphological analysis. Induction of a partial secondary axis was observed in 71% (upper middle; *N* = 45) of BVg1-injected embryos and in 68% (upper right; *N* = 47) of BVg1 and Cm-*derrière*-co-injected embryos. Thus, Cm-*derrière* did not inhibit the ability of BVg1 to induce a partial secondary axis. Bottom panels, embryos were injected with 50 pg of *derrière* mRNA or 50 pg of *derrière* mRNA plus 500 pg of Cm-*derrière* mRNA in the ventrovegetal blastomere at 8-cell stage and were harvested at stage 45 for morphological analysis. Induction of a partial secondary axis was observed in 68% (bottom left; *N* = 57) of *derrière*-injected embryos, and this effect was completely rescued by co-injection of Cm-*derrière* (bottom right; *N* = 41). An arrowhead shows a partial secondary axis. (**B** and **C**) Effect of Cm-*derrière* on the ability of BVg1 to invert the left–right orientation. Embryos were injected with 300 pg of BVg1 mRNA or 300 pg of BVg1 mRNA plus 100, 500 or 1000 pg of Cm-*derrière* mRNA in R2 or R3 at 16-cell stage and were harvested at stage 45 for observation of cardiac and visceral left–right orientation. Injection of BVg1 mRNA into R2 or R3 resulted in the reversal of cardiac orientation (middle panel, B) in 44% (*N* = 27) or in 74% (*N* = 39) of the injected embryos, respectively. Co-injection of BVg1 mRNA and Cm-*derrière* mRNA into R2 or R3 resulted in the reversal of cardiac orientation (right panel, B). The results are summarized in (C), which indicates that Cm-*derrière* can rescue the alteration of cardiac orientation induced by expression of BVg1. Cm-*derr*, Cm-*derrière*.

Because *derrière* is expressed zygotically *in vivo* (Sun *et al.*, 1999), we examined the effect of expression of *derrière* shortly after the mid-blastula transition by performing plasmid DNA injections with pCS2 and pCSKA. Both vectors gave essentially the same results. Although injection of 50 pg of WT *derrière* cDNA into R3 resulted in embryonic lethality in ~70% (total *n* = 292) of injected embryos, 41% of the surviving embryos exhibited reversed cardiac looping. As increasing the amount of cDNA caused further increase in embryonic lethality, we could not examine the effect of higher doses of WT *derrière* in the cDNA injections. Taken together, these results indicate that *derrière* can invert cardiac and visceral left–right asymmetry, like Vg1.

Expression of Cm-*derrière* on the left side partially randomizes cardiac left–right orientation

If *derrière* normally regulates left–right asymmetry, the above result implies that it should function in the left side of the embryo, like the suggested role of Vg1, and inhibition of the

function of *derrière* in the left side should lead to randomization of left–right asymmetry. To test this possibility, we injected dominant-negative *derrière* (Cm-*derrière*) RNA into the left side of 16-cell-stage embryos. Although injection of Cm-*derrière* at 4-cell stage led to posterior truncation (not shown; Sun *et al.*, 1999), embryos injected with Cm-*derrière* into one of the vegetal cells at 16-cell stage could develop normally. The Cm-*derrière* injection (250 pg) into L2 and L3 altered the orientation of cardiac looping in 16 and 23% of the injected embryos, respectively (Figure 3A). Although these reversal rates are somewhat lower than the rate of complete randomization (50%), they are much higher than those of the injection into R2 (6%, $n = 34$) or R3 (6%, $n = 33$). Moreover, co-injection of WT *derrière* into L2 or L3 rescued completely the disturbed left–right orientation induced by injection of Cm-*derrière* and led to normal left–right development (Figure 3A), indicating that the effect of expression of Cm-*derrière* is specific.

Ectopic expression of WT- or Cm-*derrière* alters *Xnr-1* expression

It has been shown that the asymmetric *nodal* expression in the left LPM is highly conserved in vertebrates and is required for left–right development. We thus examined the *derrière*-injected embryos for the expression of *Xnr-1*, a *Xenopus* homolog of *nodal*. Uninjected embryos showed the left-sided *Xnr-1* expression (Figures 2E,d and 3B). When we injected WT *derrière* into L3 (total $n = 27$), a majority of embryos (78%) showed normal *Xnr-1* expression (completely left-sided expression; Figure 2E,a) and the rest (22%) showed slight expression of *Xnr-1* on the right side and much stronger expression on the left side. In contrast, injection of WT *derrière* into R3 resulted in reversal of *Xnr-1* expression (right 75%, Figure 2E,c; bilateral 16%, left 9%; $n = 32$). Injection of WT *derrière* into R2 resulted in a complex *Xnr-1* expression pattern (total $n = 54$): bilateral expression (30%, Figure 2E,b), right-sided expression (30%), left-sided expression (26%) and no detectable expression (14%). Altered *Xnr-1* expression patterns by injection of *derrière* are correlated with the rates of cardiac and visceral reversal in the *derrière*-injected embryos.

Next, we examined the effect of Cm-*derrière* on *Xnr-1* expression. While injection of Cm-*derrière* into R3 resulted in normal *Xnr-1* expression (left 64%, right 0%, bilateral 9%, not detected 27%; $n = 22$; Figure 3B), injection of Cm-*derrière* into L2 (total $n = 44$) or L3 (total $n = 42$) resulted in marked increases of bilateral (L2, 21%; L3, 38%; Figure 3B) and right-sided (L2, 9%; L3, 14%; Figure 3B) *Xnr-1* expression. Thus, ectopic expression of Cm-*derrière* on the left side can partially randomize *Xnr-1* expression.

Taken together, these results suggest that *derrière* is involved in the molecular pathway developing the left–right asymmetry upstream of *Xnr-1*, probably functioning in the left side of the embryo, although the result that expression of Cm-*derrière* cannot induce complete randomization may suggest the existence of another factor that plays the equivalent role. We examined whether *derrière* exhibits an asymmetric expression pattern at any stages, like other asymmetrically expressed genes. We performed whole-mount *in situ* hybridization for *derrière*, but we could not detect its asymmetric expression (Figure 2F). This result does not, however, rule out the possibility of asymmetric expression in a highly localized region of the left side in a specific period. In addition, as recent studies have suggested the existence of the molecular flow producing the left–right gradient of ligands outside the cells in mouse embryos (Nonaka *et al.*, 1998; Okada *et al.*, 1999; Takeda *et al.*, 1999) and also the existence of the asymmetrically localized specific antagonists (Esteban *et al.*, 1999; Yokouchi *et al.*, 1999; Zhu *et al.*, 1999), it is possible that the activity of *derrière* is regulated by such mechanisms.

Cm-*derrière* inhibits the inversion of cardiac and visceral orientation induced by BVg1

Next we studied a possible interaction between *derrière* and Vg1. Co-injection of Cm-*derrière* (500 pg) into the ventral vegetal region of 8-cell embryos, which inhibited the WT *derrière* (50 pg)-induced formation of a partial secondary axis (Figure 4A), did not affect the ability of BVg1 (300 pg) to induce a secondary axis (Figure 4A), indicating that Cm-*derrière* acts as a specific inhibitor of the function of *derrière* and does not inhibit the activity of BVg1 directly. This is also supported by the result of the RT–PCR analysis shown in Figure 1B. In contrast, in left–right development, Cm-*derrière* rescued the alteration of the left–right orientation induced by BVg1 (Figure 4B). While injection of BVg1 (300 pg) into R2 and R3 altered the orientation of cardiac looping in 44% ($n = 36$) and 74% ($n = 39$) of injected embryos, respectively (Figure 4C), co-injection of Cm-*derrière* (500 pg) and BVg1 (300 pg) resulted in the normal left–right orientation of cardiac looping and visceral coiling (Figure 4B, right panel and 4C), only 5–8% of double-injected embryos showed the inverted phenotype (Figure 4C). The effect of co-injection of Cm-*derrière* was dose dependent (Figure 4C). These results indicate that Cm-*derrière* inhibits the ability of BVg1 to invert the left–right axis. In animal cap explants, BVg1 induced expression of *derrière* transcripts (Figure 1B). It might be possible, therefore, that *derrière* acts downstream of Vg1 in embryos. Moreover, we examined whether Cm-*derrière* inhibits the left–right reversal induced by *Xnr-1*. We injected plasmid pCSKA encoding *Xnr-1* into the dorsal right or dorsal left blastomere of 4-cell embryos. Injection of pCSKA/*Xnr-1* (50 pg) into the right side altered the orientation of cardiac looping in 68% of injected embryos, and some of them showed heterotaxia ($n = 57$). In contrast, injection of pCSKA/*Xnr-1* (50 pg) into the left side had little effect on the left–right asymmetry, and only 7% of injected embryos showed heterotaxia ($n = 54$). When we injected Cm-*derrière* (500 pg) together with pCSKA/*Xnr-1* (50 pg) into the right side, 41% of injected embryos ($n = 51$) showed reversal of cardiac looping. These results showed that Cm-*derrière* does not block significantly the left–right reversal induced by *Xnr-1*, whereas it does block almost completely the left–right reversal induced by BVg1 (Figure 4C), and supported our argument that *derrière* acts upstream of *Xnr-1*. The slight, inhibitory effect of Cm-*derrière* on left–right reversal induced by *Xnr-1* observed here is consistent with the previous report, which showed that Cm-*derrière* inhibits the activity of *Xnr-1* to some extent (Sun *et al.*, 1999), although the reason is unknown at present.

It is possible that maternal Vg1 proprotein could be processed to a functional form in the left side of embryos where it could induce *derrière* transcripts zygotically, although asymmetric

expression of the bulk of *derrière* could not be observed, and these secreted TGF-β family ligands could act cooperatively in establishing left–right asymmetry including the asymmetric *nodal* (*Xnr-1*) expression. An alternative possibility is that an endogenous left–right coordinator is *derrière* itself, and the observed ability of ectopically expressed Vg1 on the right side to invert the left–right axis might result from its ability to induce *derrière*. The idea that a zygotically expressed gene could function as a left–right coordinator is consistent with the observations that ectopic expression of BVg1 (Hyatt and Yost, 1998) or *derrière* (this study) after the mid-blastula transition also exhibits the inversion of the left–right axis. In conclusion, our results suggest that the recently identified TGF-β family ligand, *derrière*, may play an important role in early molecular events initiating and developing left–right asymmetry, although when and how the function of *derrière* is directed to the left side of the embryo remain to be elucidated.

METHODS

Plasmid construction and microinjection. Using a subtractive hybridization strategy, we isolated *derrière* as one of activin-inducible genes (H. Hanafusa, N. Masuyama and E. Nishida, in preparation). Full-length cDNA clones were obtained by screening a λZAPII cDNA library made from stage 10.5 embryos. The coding region of *derrière* or *Xnr-1* was amplified by PCR and cloned into the vector CS2 or CSKA. A cleavage-deficient mutant of *derrière* (Cm-*derrière*) was constructed according to the strategy of Hawley *et al.* (1995). The mutagenic oligonucleotide (CAATTGCAAAACTCAAGGAGTCGACGGGAGTACTCATTCATC) was used to convert the cleavage site RAKR to GVDG. *In vitro* synthesis of capped mRNA was performed using the Ambion mMESSAGE mMACHINE kit. Templates were as follows: *Not*I-linearized pCS2*derrière* and pCS2Cm-*derrière*; *Eco*RI-linearized pSP64TBVg1 (Thomsen and Melton, 1993). The RNAs were injected into 2-, 8- or 16-cell-stage embryos. The amounts of injected RNAs and sites of injection are described in the text and figure legends.

Embryo manipulations and immunoblotting. Embryos were *in vitro* fertilized, dejellied and cultured in 0.1× MBS [1.5 mM HEPES pH 7.4, 8.8 mM NaCl, 0.1 mM KCl, 0.24 mM $NaHCO_3$, 0.082 mM $MgSO_4$, 0.03 mM $Ca(NO_3)_2$ and 0.041 mM $CaCl_2$]. In anti-Myc immunoblotting experiments, embryos were injected with WT *derrière*-Myc mRNA (100 pg), Cm-*derrière*-Myc mRNA (100 pg), or WT *derrière*-Myc mRNA (100 pg) and Cm-*derrière* mRNA (1 ng) at the 2-cell stage and incubated for 12 h. Aliquots of the incubation media from 30 injected embryos and the whole-embryo extracts from five injected embryos were separated by 10% SDS–PAGE and transferred to a PVDF membrane (Millipore). Immunoblotting was performed using the 9E10 anti-Myc antibody (Santa Cruz Biotechnology) and visualized by the ECL western blotting detection system (Amersham).

Oocyte injections and RT–PCR analysis. Oocytes were surgically removed from females, manually defolliculated by digestion with 1.5 mg/ml collagenase in OR2 (5 mM Tris–HCl pH 7.8, 82.5 mM NaCl, 2.5 mM KCl, 1 mM Na_2HPO_4), injected with 20 ng of *in vitro* transcribed mRNA and cultured at 19°C in OR2 supplemented with 1 mM $MgCl_2$, 1 mM $CaCl_2$ and 0.5 mg/ml bovine serum albumin (BSA). Conditioned media from oocytes were prepared by incubating oocytes in OR2 with $MgCl_2$, $CaCl_2$ and BSA for 3 days. For RT–PCR analysis, animal cap explants were prepared at stage 8 and cultured in 0.1× MBS supplemented with the oocyte conditioned media until sibling embryos reached stage 11 (early) or stage 26 (late). Total RNA was isolated from six animal cap explants or five whole embryos using TRIzol (Gibco-BRL) according to the manufacturer's instructions. PCR conditions were: 94°C, 30 s; 58°C, 30 s; 72°C, 30 s; for 25 cycles (EF1α and *derrière* in Figure 1C for 20 cycles). Primers for *Xbra*, *gsc*, *EF1α* and *muscle-actin* were as described (Masuyama *et al.*, 1999). The sequences of other primer pairs used were as follows: *derrière* forward 5′-ATATTATGGACAACAGTTCC-3′, reverse 5′-ACTACAAATGATCGATTGCC-3′; *derrière* 3′UTR forward 5′-GTTTGCTTTGGAGATTGTTC-3′, reverse 5′-TGTTTCATCCAGCAGCTCTG-3′; *XlHbox6* forward 5′-GCAATCTGAACCCCTGGACT-3′, reverse 5′-TTAGTGTCGGGGAAGTTGCC-3′.

***In situ* hybridization and histological analysis.** Injected embryos were fixed at stage 22–24 by MEMFA (0.1 M MOPS pH 7.4, 2 mM EGTA, 1 mM $MgSO_4$, 3.7% formaldehyde) treatment for whole-mount *in situ* hybridization and histological sectioning. Whole-mount *in situ* hybridization was performed as described (Masuyama *et al.*, 1999). For histology, fixed embryos were dehydrated through ethanol–xylene series, embedded in paraffin, and 7 μm sections were cut on a rotary microtome. Sections were stained with hematoxylin–eosin (Sigma) according to the manufacturer's protocol.

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