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The roles of three signaling pathways in the formation and function of the Spemann Organizer

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SUMMARY

Since the three main pathways (the Wnt, VegT and BMP pathways) involved in organizer and axis formation in the *Xenopus* embryo are now characterized, the challenge is to understand their interactions. Here three comparisons were made. Firstly, we made a systematic comparison of the expression of zygotic genes in sibling wild-type, VegT-depleted (VegT$^-$), β-catenin-depleted (β-catenin$^-$) and double depleted (VegT$^-$/β-catenin$^-$) embryos and placed early zygotic genes into specific groups. In the first group some organizer genes, including *chordin*, *noggin* and *cerberus*, required the activity of both the Wnt pathway and the VegT pathway to be expressed. A second group including *Xnr1, 2, 4* and *Xlim1* were initiated by the VegT pathway but their dorsoventral pattern and amount of their expression was regulated by the Wnt pathway. Secondly, we compared the roles of the Wnt and VegT pathways in producing dorsal signals. Explant co-culture experiments showed that the Wnt pathway did not cause the release of a dorsal signal from the vegetal mass independent from the VegT pathway. Finally we compared the extent to which inhibiting Smad 1 phosphorylation in one area of VegT$^-$, or β-catenin$^-$ embryos would rescue organizer and axis formation. We found that BMP inhibition with *cm-BMP7* mRNA had no rescuing effects on VegT$^-$ embryos, while *cm-BMP7* and *noggin* mRNA caused a complete rescue of the trunk, but not of the anterior pattern in β-catenin$^-$ embryos.

Key words: VegT, β-catenin, Wnt, TGFβ, Organizer, Antisense oligo, Morpholino

INTRODUCTION

Three major signaling pathways have been shown to be essential in establishing mesodermal pattern, the Wnt/β-catenin, BMP/Smad1 and VegT/Xnr-derriere/Smad 2 pathways. The vegetally localized, maternal T-box transcription factor, VegT was shown to induce ventral, general and dorsal mesoderm by regulating the expression of activin-like TGFβs, *derriere*, *Xnr1, 2, 4, 5* and *6*, as well as *chordin*, *cerberus* and zygotic *FGF*s (Zhang et al., 1998; Kofron et al., 1999; Takahashi et al., 2000). Over-expression and indirect loss-of-function experiments indicated that Xnrs were particularly important mesoderm inducers (Osada and Wright, 1999; Agius et al., 2000; Takahashi et al., 2000; Tanegashima et al., 2000). Many studies suggested that the interaction of a TGFβ pathway in the dorsal vegetal quadrant with a dorsal Wnt pathway could induce organizer gene expression (Steinbeisser et al., 1993; Watabe et al., 1995; Carnac et al., 1996; Crease et al., 1998; Agius et al., 2000; Nishita et al., 2000). Jones et al. first described the expression of *Xnr*s (Jones et al., 1995). Agius et al. showed that *Xnr* mRNAs exist in concentration gradients from dorsal to ventral across the vegetal mass of the late blastula, and that the Wnt and VegT pathways synergized to produce the high dorsal expression (Agius et al., 2000). However, Lee et al. did not find a simple static gradient of Smad 2 phosphorylation, evidence of an active Xnr-mediated signaling pathway, in the blastula. They demonstrated an earlier onset of Smad2 signaling dorsally than ventrally, a difference that was lost in ultraviolet radiation (uv)-ventralized embryos (Lee et al., 2001).

The Wnt signaling pathway may also play complex roles in patterning the mesoderm. Recent immunostaining studies showed that nuclear β-catenin, evidence of the transduction of Wnt signals, was not restricted to the dorsal side of the blastula, but was also found ventrolaterally and vegetally, during the late blastula stage (Schohl and Fagotto, 2002). Also, targeted depletion experiments that removed *β-catenin* mRNA from different quadrants of the early embryo suggested that there may be multiple functions for the Wnt signaling pathway in the early embryo (Heasman et al., 2000). At least four transcription factors of the HMG box class that bind β-catenin have been shown to be expressed in the late blastula and have the potential to be regulated by β-catenin (Molenaar et al., 1998; Zorn et al., 1999a; Houston et al., 2002).

Table 1. A summary of studies in which ectopic axes were formed by blocking BMP activity

Reference	Blocking reagent	Description of partial secondary axis
Graff et al., 1994	tBR	Lacked organized head structures and notochord
Hawley et al., 1995	Cm-XBMP7 Cm-XBMP4	Lacked head structures, contained neural tube and notochord
Ishikawa et al., 1995	Human tBMPRII	Lacked anterior structures, contained neural and endodermal tissues
Schmidt et al., 1995	ΔTmTFR11	
Suzuki et al., 1995	ΔTmTFR11	Neural and muscle tissue induced without notochord.
Frisch et al., 1998	tBRII (also tBR for comparison)	tBRII – absence of visible floor plate and notochord, contained some muscle. Sometimes anterior structures seen. Secondary axis extends more anteriorly than when second axis is induced by tBR
Yamamoto et al., 2001	HI-Xmsx1	Ectopic head including eye, cement gland, notochord, primary axis significantly shortened
	TI-Xmsx1	Partial secondary axis with no head or notochord
Yasuo and Lemaire 2001	tBR	Neural tube and somites. No notochord

The original three-signal model suggested that an early ventral signal from the vegetal mass specified ventral mesodermal fates (Smith, 1989). Although ectopically expressed BMP could induce ventral mesoderm formation (Dale et al., 1992; Jones et al., 1992; Fainsod et al., 1994), making it a candidate for the ventral mesoderm inducer, BMP acted as an indirect inhibitor of *XFD1* (Friedle et al., 1998), and of nodal signaling via *Xmsx1* (Yamamoto et al., 2001), making it more likely to act as an inhibitor of organizer fates, than as a specifier of ventral mesoderm. Furthermore, Smad1 signaling activity was not ventrally localized, but quite uniform over the vegetal hemisphere until the onset of gastrulation (Faure et al., 2000; Schohl and Fagotto, 2002).

How then do these signaling pathways interact to specify the organizer region?

The aim here was first to ask whether key early zygotic genes fell into specific groups; those that required the activity of both the Wnt pathway and the VegT pathway to be expressed, versus those that require only one of the pathways, or neither of the pathways. We made a systematic comparison of the expression of zygotic genes in sibling wild-type, VegT-depleted (VegT$^-$), β-catenin-depleted (β-catenin$^-$) and double depleted (VegT$^-$/β-catenin$^-$) embryos. No direct comparison has been carried out before and the temporal series was important to distinguish whether genes were delayed in the onset of expression or off altogether in the 12-hour period studied. Although we have previously studied the phenotype of VegT$^-$ embryos in some depth (Zhang et al., 1998; Kofron et al., 1999; Xanthos et al., 2001), β-catenin$^-$ embryos have received less attention (Heasman et al., 1994; Wylie et al., 1996; Heasman et al., 2000). Studies using uv irradiation to ventralize embryos (Fainsod et al., 1994; Graff et al., 1994; Steinbeisser et al., 1995; Bouwmeester et al., 1996; Darras et al., 1997; Lee et al., 2001) have the disadvantage that uv does not phenocopy β-catenin depletion, making the experiments difficult to interpret.

Secondly, we used β-catenin$^-$ embryos to study the nature of the organizer-inducing activity that comes from the vegetal mass of the blastula (Nieuwkoop, 1969; Kofron et al., 1999; Agius et al., 2000). We showed that β-catenin$^-$ equators (that express general mesodermal, but not dorsal mesodermal markers) could not be rescued by VegT$^-$ vegetal masses, indicating that there was no measurable endogenous dorsal vegetal signal that was independent of the VegT/TGFβ signaling pathway.

Thirdly, we examined the importance of Wnt signaling in regulating *Xnr* expression. We found that *Xnr1*, *2* and *4* mRNAs had similar dorsoventral expression patterns and that in β-catenin$^-$ embryos, the dorsal/ventral differences were lost, and the amount of expression of these Xnrs was reduced.

Lastly, since we had shown that the Wnt pathway and the VegT pathway were both essential for activating the expression of organizer genes involved in antagonizing BMP signaling, we addressed the importance of this antagonism for axis formation. Inhibition of endogenous Smad1 phosphorylation by a variety of means has been shown to cause different degrees of ectopic axis formation (Table 1). We previously blocked BMP signaling using (1) a truncated BMP receptor, *tBR* mRNA, in maternal β-catenin-depleted embryos, and rescued the formation of a headless axis, and (2) with *noggin* mRNA, rescued a more complete axis (Wylie et al., 1996). In uv-irradiated embryos, blocking BMP signaling using *noggin* or *chordin* mRNA rescued axis formation completely (Smith and Harland, 1992; Sasai et al., 1994). These variations in the type of rescued axis might have arisen because of varying degrees of influence from the endogenous axis, or because of differences in the mode of BMP blockade.

Here we used embryos depleted of maternal β-catenin or VegT, and rescued with a cleavage mutant form of BMP7, *cm-BMP7* mRNA (Hawley et al., 1995) that we showed specifically inhibited endogenous Smad1, but not Smad2, phosphorylation. We found that BMP inhibition had no rescuing effects on VegT$^-$ embryos, indicating that VegT targets were essential for axis formation. In comparison, BMP inhibition caused a complete rescue of the trunk, but not the anterior pattern of β-catenin$^-$ embryos. Since many reports have shown that the repression of zygotic Wnt signaling was necessary for anterior neural differentiation (Baker et al., 1999; Erter et al., 2001; Hartley et al., 2001; Kiecker and Niehrs, 2001; Lekven et al., 2001; Yao and Kessler, 2001), we also blocked zygotic, together with maternal, Wnt signaling, using a morpholino oligo. This improved anterior axis formation, but normal head, heart and anterior gut formation was not restored. Similarly, the secreted BMP antagonist *noggin* mRNA was unable to restore normal anterior pattern. These results are summarized diagrammatically.

MATERIALS AND METHODS

Oocytes and embryos

Full-grown oocytes were manually defolliculated and cultured in oocyte culture medium (OCM), as described previously (Zuck et al., 1998). Oocytes were injected at the vegetal pole with oligo using a

Medical Systems picoinjector, in OCM and cultured for a total of 24-48 hours at 18°C before fertilization. In preparation for fertilization, oocytes were stimulated to mature by the addition of 2 μM progesterone to the OCM and cultured for 12 hours. Oocytes were then colored with vital dyes and fertilized using the host-transfer technique described previously (Zuck et al., 1998). Three hours after being placed in the frog's body cavity, the eggs were stripped and fertilized along with host eggs using a sperm suspension. Embryos were maintained in 0.2× MMR, and experimental embryos were sorted from host embryos. Unfertilized eggs and abnormally cleaving embryos were removed from all batches.

Injections into embryos were carried out in 1% Ficoll in 0.5× MMR pH7.6 as described in the text. For fate mapping experiments, *cm-BMP7* mRNA or *noggin* mRNA was diluted 1:1 with *lacZ* mRNA at 100 pg/nl concentration, so that 200 pg of *lacZ* mRNA was injected along with 300-600 pg *cm-BMP7* or 1-50 pg of *noggin* mRNA. Embryos were washed thoroughly and returned to 0.2× MMR during the blastula stage.

Explant culture

Mid-blastula stage-8 wild-type, VegT$^-$, β-catenin$^-$ and VegT$^-$/β-catenin$^-$ embryos were devitellined and dissected with tungsten needles as shown in Fig. 2A and Fig. 3A on agar-coated dishes in 1× MMR. After washing away dead cells, vegetal and equatorial pieces were placed together in the combinations described in Fig. 2B and Fig. 3A and cultured on agar in OCM for 2 hours. The recombinants were separated using tungsten needles and stray vegetal cells were identified by their different vital dye coloring and removed from the explants. Equatorial explants were then cultured in fresh OCM on agar until sibling uninjected embryos were stage 11 or 16 as described in the text, photographed and frozen in batches of three equators for analysis.

For dissections into dorsal and ventral halves, the dorsal side of embryos was marked at the four-cell stage, using Nile Blue crystals. The dorsoventral axis was recognized at the four-cell stage by the pigmentation differences of the dorsal and ventral sides. When wild-type embryos reached stage 9, all the batches were placed on 2% agar dishes in 1× MMR, pH 7.6 and bisected into dorsal and ventral halves, at 2-hourly intervals through the late blastula and gastrula stages, and frozen in groups of 4 half-embryos.

Oligos and mRNAs

The antisense oligodeoxynucleotides used were HPLC purified phosphorothioate-phosphodiester chimeric oligonucleotides (Sigma/Genosys) with the base composition:

VegT C*A*G*CAGCATGTACTT*G*G*C
β-catenin T*G*C*C*TTTCGGTCTG*G*C*T*C,

where * represents a phosphorothioate bond. The morpholino β-catenin oligo was a 25 mer (Gene Tools LLC) with the base composition: 5′-TTTCAACCGTTTCCAAAGAACCAGG-3′.

Oligos were resuspended in sterile, filtered water and injected in doses as described in the text. Capped *cm-BMP7* mRNA was synthesized using the MEGAscript kit (Ambion) from pSP64T vector, linearized with *Xho*I, transcribed by SP6, ethanol precipitated and resuspended in sterile distilled water for injection.

Fixation and histology

For X-gal staining, embryos were fixed in MEMFA for 2 hours, rinsed in PBS and stained using X-gal. Embryos were washed in PBS after staining and photographed before clearing with Murray's clear (2:1 butyl alcohol and butyl butyrate). Embryos were bleached with a 3% hydrogen peroxide solution. For histology, embryos were dehydrated, and embedded in low-melt wax, serially sectioned at 20 μm and stained with Hematoxylin and Eosin.

Western blot analysis

Western blot analysis with anti-phospho Smad2 antibody was used after affinity purification from crude antisera (Peter ten Dijke), and using anti-Smad2 antibody from Transduction Labs. Secondary antibodies were goat anti-rabbit IgG-HRP antibodies (Boehringer Mannheim) and donkey anti-mouse IgG-HRP F(ab)$_2$ fragments (Jackson Immunoresearch Laboratories). Western analysis was carried out as described by Lee et al. (Lee et al., 2001).

Analysis of gene expression using real time RT-PCR

Real-time RT-PCR, rather than radioactive RT-PCR or northern blot analysis, was used to analyze gene expression in wild-type and depleted embryos, since side by side comparisons of radioactive PCR versus real-time PCR showed that the latter has major advantages. The method has higher sensitivity, both in terms of detecting non-abundant mRNAs and in terms of the amount of cDNA needed for each run. Also by doing a melting curve analysis after each reaction we could ensure that only one product was amplified. Many published primer pairs amplified multiple products as determined by melting curve analysis, and so could not be used for this comparative analysis. To determine the reproducibility of the technique we compared replicates of embryos, RNA and cDNA. This allowed us to test the fidelity of the RNA extraction, cDNA synthesis, and PCR amplification, respectively. We found that real-time PCR on duplicate samples gave no more than 4% variation on identical samples. Real-time PCR on separate cDNA made from the same RNA showed no more than 7% variation between samples. Real-time PCR on cDNA made from RNA extracted from identically treated embryos showed no more than 13% variation between replicates.

Total RNA was prepared from oocytes and embryos using the proteinase K method as described previously (Kofron et al., 1999). The primers used and cycling conditions are listed in Table 2. In order to compare expression levels of depleted and rescued embryos relative to controls, a dilution series of uninjected control cDNA was made and assayed in each LightCycler run (Roche). Undiluted control cDNA=100%, 1:1 cDNA: H_2O=50% and 1:10 cDNA: H_2O=10%. In experiments where multiple embryonic stages were examined, the dilution series was used from cDNA of the uninjected control stage of development predicted to give the highest expression of the gene product being amplified. These values were entered as concentration standards in the LightCycler sample input screen. Other controls included in each run were –RT and water blanks. These were negative in all cases but not included in the figures for lack of space. LightCycler quantification software v1.2 was used to compare amplification in experimental samples during the log-linear phase to the standard curve from the dilution series of control cDNA. The comparisons were displayed as histograms. In each case, *ornithine decarboxylase* (ODC) was used as a loading control (data not shown), and each bar was normalized to the level of ODC expression.

For each primer pair used, we optimized conditions so that melting curve analysis showed a single melting peak after amplification, indicating a specific product.

RESULTS

The relative importance of Wnt signaling compared to VegT signaling for the expression of key zygotic genes

The developmental series used here consisted of embryos frozen at 2-hourly intervals from the time of initiation of zygotic gene expression (stage 8 blastula), to the end of gastrulation (stage 11.5). This allowed us to distinguish genes that were delayed in their onset of expression from those that were not expressed. We placed genes in the 'not expressed' category if their level of expression over the 12-hour period of study never rose above 10% of control levels. The

Table 2. Primer pairs and PCR cycling conditions used with the LightCycler™

PCR primer pair	Reference	Sequence	Denat. temp°C	Anneal temp°C/ time (sec)	Extension temp°C/ time (sec)	Acquisition temp°C/ time (sec)
Antipodian	Stennard et al., 1999	U: 5′-TGG ATT AGT TTA GGA ACA-3′ D: 5′-CGG ATC TTA CAC TGA GGA-3′	95	55/5	72/16	83/3
Bmp4	New	U 5′-ACC CAT AGC TGC AAA TGG AC-3′ D 5′-CAT GCT TCC CCT GAT GAG TT-3′	95	55/5	72/12	81/3
Cerberus	Heasman et al., 2000	U: 5′-GCT TGC AAA ACC TTG CCC TT-3′ D: 5′-CTG ATG GAA CAG AGA TCT TG-3′	95	60/5	72/20	81/3
Chordin	XMMR	U: 5′-AAC TGC CAG GAC TGG ATG GT-3′ D: 5′-GGC AGG ATT TAG AGT TGC TTC-3′	95	55/5	72/12	81/3
Cresent	New	U: 5′-CTG GAC TTC TTT GGC AGC TC-3′ D: 5′-AGA CTG CGA CAT GGG AAG AT-3′	95	57/5	72/12	83/3
Derriere	Kofron et al., 1999	U: 5′-TGG CAG AGT TGT GGC TAT CA-3′ D: 5′-CTA TGG CTG CTA TGG TTC CTT-3′	95	55/5	72/18	82/3
FGF8	Kofron et al., 1999	U: 5′-CTG GTG ACC GAC CAA CTA AG-3′ D: 5′-ACC AGC CTT CGT ACT TGA CA-3′	95	55/5	72/14	86/3
Goosecoid	New	U: 5′-TTC ACC GAT GAA CAA CTG GA-3′ D: 5′-TTC CAC TTT TGG GCA TTT TC –3′	95	55/5	72/11	82/3
Mixer	Henry and Melton, 1998	U: 5′-CAC CAG CCC AGC ACT TAA CC-3′ D: 5′-CAA TGT CAC ATC AAC TGA AG-3′	95	55/5	72/12	83/3
MyoD	Rupp et al., 1991	U: 5′-AGC TCC AAC TGC TCC GAC GGC ATG AA-3′ D: 5′-AGG AGA GAA TCC AGT TGA TGG AAA CA-3′	95	55/5	72/18	86/3
ODC	Heasman et al., 2000	U: 5′-GCC ATT GTG AAG ACT CTC TCC ATT C-3′ D: 5′-TTC GGG TGA TTC CTT GCC AC-3	95	55/5	72/12	83/3
Plakoglobn	Kofron et al., 1999	U: 5′-GCT CGC TGT ACA ACC AGC ATT C-3′ D: 5′-GTA GTT CCT CAT GAT CTG AAC C-3′	95	60/10	72/16	85/3
Noggin	XMMR	U: 5′-AGT TGC AGA TGT GGC TCT-3′ D: 5′-AGT CCA AGA GTC TGA GCA-3′	95	55/5	72/12	84/3
Siamois	Heasman et al., 2000	U: 5′-CTG TCC TAC AAG AGA CTC TG-3′ D: 5′-TGT TGA CTG CAG ACT GTT GA-3′	95	55/5	72/16	81/3
Sizzled	New	U: 5′-CAC ACA AGA CAG TCT TGG AAG CTT TC-3′ D: 5′-CAC CAG CAA TAA CAT ACA CTG TGG G-3′	95	54/5	72/10	81/1
Xbra	Sun et al., 1999	U: 5′-TTC TGA AGG TGA GCA TGT CG-3′ D: 5′-GTT TGA CTT TGC TAA AAG AGA CAG G-3′	95	55/5	72/8	75/3
XHex	Chang et al., 2000	U: 5′-AAC AGC GCA TCT AAT GGG AC-3′ D: 5′-CCT TTC CGC TTG TGC AGA GG-3′	95	60/5	72/13	87/3
Xlim1	New	5′-CCCTGGCAGCAACTATGACT-3′ 5′-GGTTGCCATAACCTCCATTG-3′	95	55/5	72/11	85/3
Xnr1	Kofron et al., 1999	U: 5′-TGG CCA GAT AGA GTA GAG-3′ D: 5′-TCC AAC GGT TCT CAC TTT-3′	95	55/5	72/12	81/3
Xnr3	Kofron et al., 1999	U: 5′-CTT CTG CAC TAG ATT CTG-3′ D: 5′-CAG CTT CTG GCC AAG ACT-3′	95	57/5	72/10	79/3
Xnr6	Takahashi et al., 2000	U: 5′-TCC AGT ATG ATC CAT CTG TTG C-3′ D: 5′-TTC TCG TTC CTC TTG TGC CTT-3′	95	55/5	72/10	83/3
Xsox17	Xanthos et al., 2001	U: 5′-GCA AGA TGC TTG GCA AGT CG-3′ D: 5′-GCT GAA GTT CTC TAG ACA CA-3′	95	58/5	72/8	85/3
Xvent1	Gawantka et al., 1995	U: 5′-GCA TCT CCT TGG CAT ATT TGG-3′ D: 5′-TTC CCT TCA GCA TGG TTC ACC-3′	95	62/5	72/20	83/3
Xwnt8	Ding et al., 1998	U: 5′-CTG ATG CCT TCA GTT CTG TGG-3′ D: 5′-CTA CCT GTT TGC ATT GCT CGC-3′	95	58/6	72/14	85/3

experiment was repeated on a second developmental series with the same result. Sibling embryos of those tested were allowed to develop to the tail-bud stage and all displayed severe phenotypes (data not shown). We used two oligos directed against β-catenin, one a morpholino oligo (Heasman et al., 2000), which depleted both maternal and zygotic β-catenin protein, and a thioate chimeric oligo, which depleted only maternal *β-catenin* mRNA (Heasman et al., 1994). These embryos were indistinguishable from each other in appearance (see Fig. 6A) and showed no significant differences in the expression of molecular markers at the gastrula stage. Therefore, only the results for the morpholino β-catenin$^-$ embryos were shown.

The genes studied here were of different: types organizer (*gsc*, *Xlim1*, *chordin*, *siamois*, *Xnr3*, *Xnr1*, *Xnr6*, *noggin*, *cerberus*, *Xhex*, *crescent*, *Xlim1*), general mesoderm (*Apod*,

Xbra, *FGF8*), ventrolateral mesoderm (*Xwnt8*, *BMP4*, *Xvent1*, *sizzled*) and endoderm (*Xsox17α*, *mixer*, *derriere*).

We found that gastrula genes could be placed into 4 categories:

Group1: genes requiring both Wnt/β-catenin and VegT pathways for expression

Fig. 1A shows those genes that were not expressed if either β-catenin or VegT was depleted. They included the homeobox gene *gsc* and those of the secreted proteins Chordin, Cerberus, Crescent and Noggin. In both β-catenin$^-$ and VegT$^-$ embryos, these genes were not expressed or were expressed at less than 10% control levels throughout the blastula and gastrula stages. *Xnr6* were also expressed at a maximum of 15% of control levels.

Group 2: Genes initiated by Wnt/β-catenin pathway activity

Fig. 1B shows the expression pattern of the Wnt target genes *siamois* and *Xnr3*. As shown by others, in wild-type embryos, *siamois* and *Xnr3* expression peaked at stage 9. In β-catenin$^-$ embryos, they were not expressed throughout the temporal series, while in VegT$^-$ embryos their expression peaked 2 hours later and at a much lower level compared to controls (20% control level for *siamois*; and 30% control levels for *Xnr3*). Although several attempts were made to analyse *Xtwin*, a *siamois*-related gene (Laurent et al., 1997) real-time RT-PCR consistently produced several products in melting curve analysis, with 5 primer pairs including the published ones, suggesting that several forms of the mRNA were being amplified, and so no histograms could be generated.

Group 3: genes initiated by VegT

Fig. 1C shows the organizer, endodermal and general mesodermal genes dependent on VegT but not β-catenin for expression. These included organizer genes *Xlim1*, *Xhex* and *Xnr1*, endodermal genes *Xsox17*, *mixer* and *derriere* and mesodermal genes *Xwnt8*, *Apod* and *Xbra*. With the exception of *derriere* and *Xwnt 8*, all these genes had reduced levels of expression in β-catenin$^-$ embryos, suggesting that the Wnt pathway modulates their expression but does not initiate it.

Group 4: genes requiring neither VegT nor the Wnt pathway for expression

Several genes, including *sizzled* and *BMP4* (Fig. 1D) continued to be expressed in both VegT$^-$ and β-catenin$^-$ embryos. In both VegT$^-$ and β-catenin$^-$ embryos, BMP4 expression began at the late blastula stage, as in wild-type siblings, and continued to be expressed at control levels until stage 11. The peak of expression at stage 11 was significantly lower in VegT$^-$ and β-catenin$^-$ embryos than in control embryos.

We compared these temporal expression patterns with those described elsewhere for these key genes in wild-type embryos, or embryos treated with uv, or other ventralizing agents, and also embryos with the VegT pathway inactivated using nodal signaling blockers. Important differences include:

(A) β-catenin$^-$ embryos did not phenocopy uv ventralized embryos. Several authors have shown that *siamois, Xnr3* and *cerberus* were ectopically expressed in uv-ventralized embryos (Brannon and Kimelman, 1996; Cui et al., 1996; Darras et al., 1997). These genes were not expressed in β-catenin$^-$ embryos (Fig. 1A,B). Uv-ventralization has been shown to increase *BMP4* mRNA expression (Fainsod et al., 1994). We found here it was not raised by either VegT or β-catenin depletion. This was also true for *BMP7* mRNA (data not shown).

(B) Dominant negative experiments that blocked nodal signaling showed that the blastula stage expression of *chordin*, *noggin* and *cerberus* was *Xnr* independent (Wessely et al., 2001). In contrast, we found here that all three were off in VegT depleted embryos.

(C) *Xhex* has been suggested to be an inducer of *cerberus* and both have a similar expression pattern (Zorn et al., 1999b; Brickman et al., 2000) However, we found that in β-catenin$^-$ embryos, *Xhex* continued to be expressed at reduced levels, but *cerberus* was not induced. This suggests that *Xhex* only induces *cerberus* when it is expressed at high levels, or that another β-catenin-dependent factor is necessary in conjunction with *Xhex*.

(D) Expression of *Xwnt8* has been shown in overexpression and dominant negative experiments to be regulated by BMP signals (Hoppler and Moon, 1998; Marom et al., 1999; Yasuo and Lemaire, 2001). Here we showed that its endogenous expression was dependent on the VegT pathway, suggesting that this pathway directly or indirectly activated *Xwnt8*. *Xwnt8* was off in VegT$^-$ embryos in which *BMP4* was expressed at normal levels.

(E) An important conclusion was that the genes for organizer proteins responsible for blocking BMP and Xnr signaling ie. *noggin*, *chordin*, *cerberus* and *crescent* were only expressed if both VegT and β-catenin pathways were functioning. In contrast, other organizer secreted proteins, *Xnrs1*, *2* and *4* were expressed in β-catenin$^-$ embryos, albeit at a lower level and without their normal pattern.

(F) The order of expression in wild-type embryos of key genes for secreted proteins was that *Xnrs*, particularly *Xnr6*, *3* and *1* were expressed first, with a peak in expression at stage 9, followed by the onset of expression of *noggin*, *chordin cerberus*, *derriere* and *FGF8* at stage 9, peaking at stage 11.

Do the VegT and Wnt pathway act separately in the vegetal mass to produce a mesodermal signal and a dorsal signal respectively?

Although much attention has recently focused on the nodal-related proteins as the most likely vegetal signals inducing organizer formation, several studies have suggested that separate dorsal-inducing and mesoderm-inducing signals exist in the vegetal mass (Wylie et al., 1996; Darras et al., 1997). An alternative view is that the influence of the Wnt pathway was not in producing its own independent secreted dorsalizing signal. To distinguish whether the vegetal mass had in fact two components, a dorsal signal, downstream of Wnt, and a mesoderm-inducing one, downstream of VegT, we dissected equatorial regions of β-catenin$^-$ embryos at the mid blastula stage and co-cultured them with wild-type, VegT$^-$ or β-catenin$^-$ vegetal masses as shown in Fig. 2A,B. The rationale for using β-catenin$^-$ equators rather the more usual wild-type animal caps was firstly, that this is the region that normally forms the organizer. Secondly, we knew that β-catenin$^-$ equators already expressed general and ventral mesodermal markers (*Xbra, Xwnt8, FGF8;* data not shown); if all that they required to form an organizer was a dorsal signal, then VegT$^-$ vegetal masses should rescue organizer formation. The equatorial/vegetal co-

cultures were separated after two hours. Vegetal cells were recognized by their vital dyes and carefully pulled away from the equators using tungsten needles. The equators were cultured until siblings reached the mid-gastrula stage, for the analysis of dorsal mesodermal markers, and the mid-neurula stage, for the examination of convergence extension movement and late mesodermal markers. Control wild-type, β-catenin⁻ and VegT⁻ equators that were untreated, or that had been co-cultured with wild-type vegetal masses were also cultured until the mid-gastrula and mid-neurula stages (not shown).

By the neurula stage, wild-type equators showed extensive convergence extension movements, while β-catenin⁻ and VegT⁻ equators did not (Fig. 2C and data not shown). Co-culture with wild-type vegetal masses rescued convergence extension movements in β-catenin⁻ equators (third column Fig. 2C). However, β-catenin⁻ equators were not rescued when VegT⁻ or β-catenin⁻ vegetal masses were used instead of wild-type ones (Fig. 2C; fourth and fifth columns). We concluded that convergence extension movements in the equatorial zone of β-catenin⁻ embryos could only be activated by vegetal induction when both Wnt and VegT components were present in the vegetal mass.

We confirmed this result by examining molecular markers of organizer formation, *chordin*, *gsc*, *siamois*, *Xnr3* and *Xnr1* in equatorial explants from the same experiment, frozen at the mid-gastrula stage (stage 11). We also examined the expression of the dorsal mesodermal differentiation marker *MyoD* in explants frozen at the neurula stage. We found that wild-type uninjected equatorial explants expressed all of the organizer markers tested, while VegT⁻ explants expressed only *siamois* and *Xnr3*, and β-catenin⁻ explants expressed only *Xnr1* (Fig. 2D). In comparison, β-catenin⁻ equators that had been co-cultured with wild-type vegetal masses, expressed mesodermal markers, *chordin*, *goosecoid* and *MyoD*, but did not express *siamois* and *Xnr3*. β-catenin⁻ equators co-cultured with VegT⁻ vegetal masses did not express *goosecoid*, *chordin*, *siamois*, *Xnr3* or *MyoD* (Fig. 2D). This experiment was repeated three times, with the same result.

These experiments supported the hypothesis that there was no measurable endogenous dorsal signal secreted by the vegetal mass over the late blastula-early gastrula stage that was independent of the VegT signaling pathway.

To determine whether the Wnt component controlling *goosecoid* and *chordin* expression had to occur with the

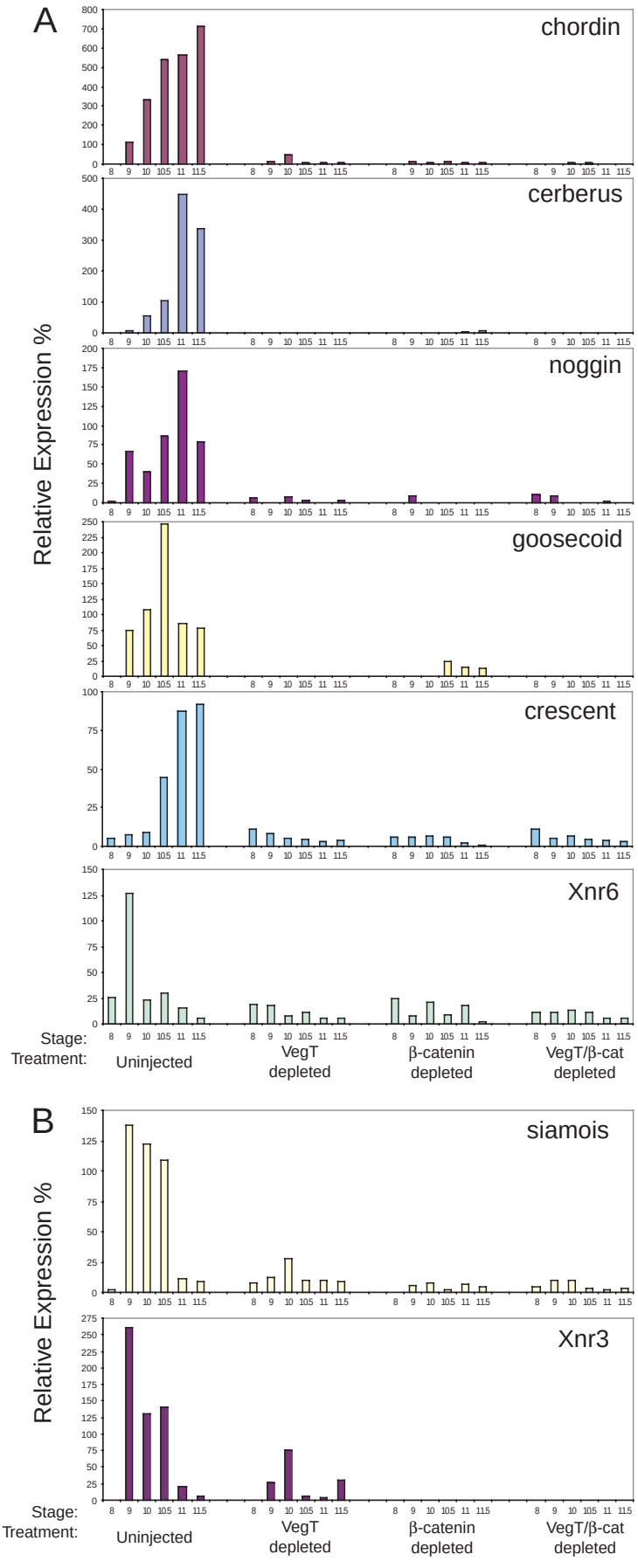
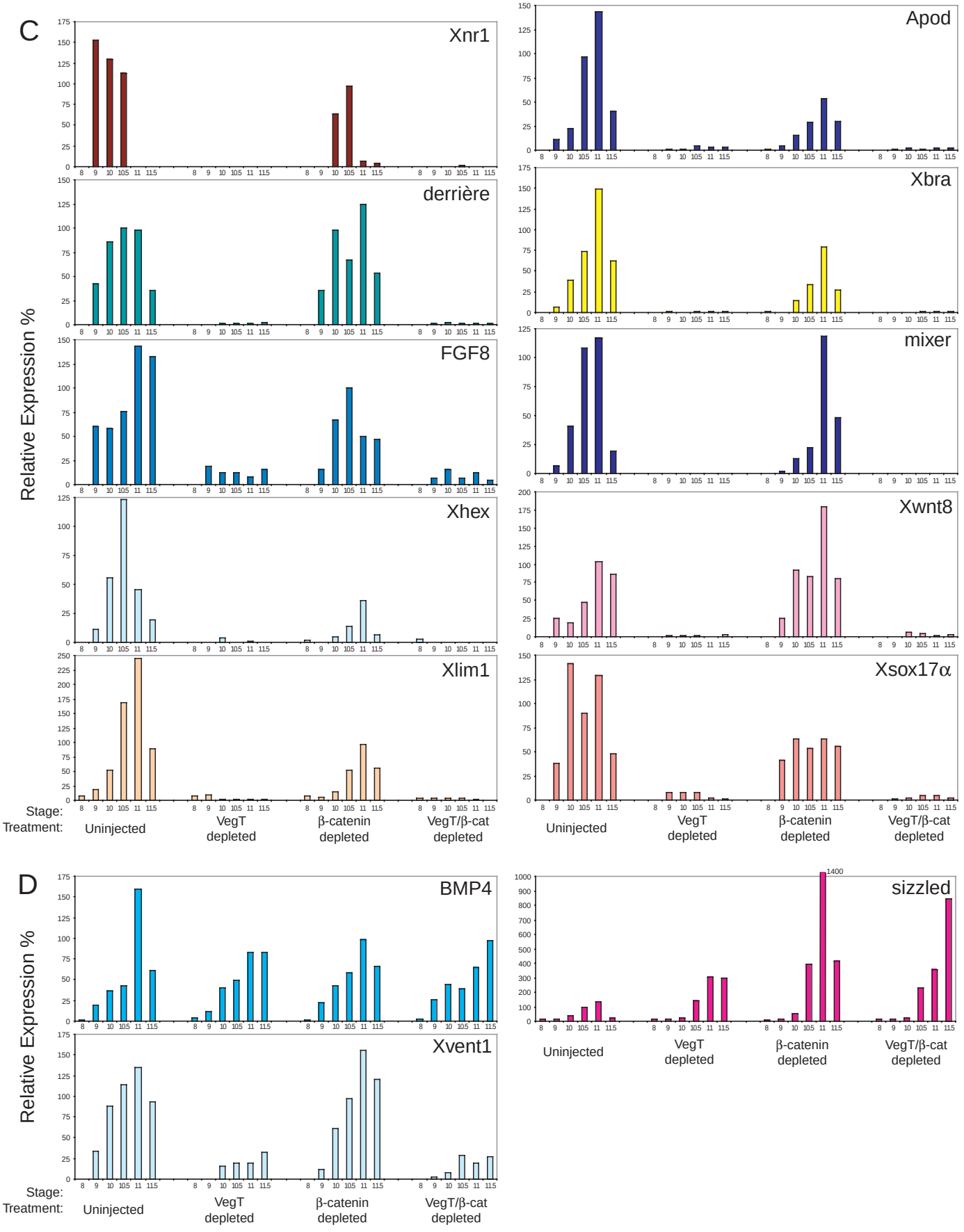


Fig. 1. The regulation of zygotic transcription by both the VegT and the Wnt pathway. Wild-type, VegT⁻, β-catenin⁻ and VegT⁻/β-catenin⁻ embryos were frozen at 2-hourly intervals from the mid-blastula (stage 8) to the late gastrula (stage 11.5) stages and analysed by real-time RT-PCR. The results are displayed as histograms to show the relative levels of expression of molecular markers representing (A) genes that require both pathways for the initiation of their expression, (B) direct targets of the Wnt pathway, (C) genes that require VegT but not β-catenin for the initiation of their expression, (D) genes activated normally in both VegT⁻ and β-catenin⁻ embryos. In each case, *ornithine decarboxylase* (ODC) is used as a loading control (data not shown), and each bar is normalized to the level of ODC expression.


C
Relative Expression %
Xnr1
derrière
FGF8
Xhex
Xlim1
Apod
Xbra
mixer
Xwnt8
Xsox17α
Stage:
Treatment:
Uninjected
VegT depleted
β-catenin depleted
VegT/β-cat depleted
D
Relative Expression %
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Stage:
Treatment:
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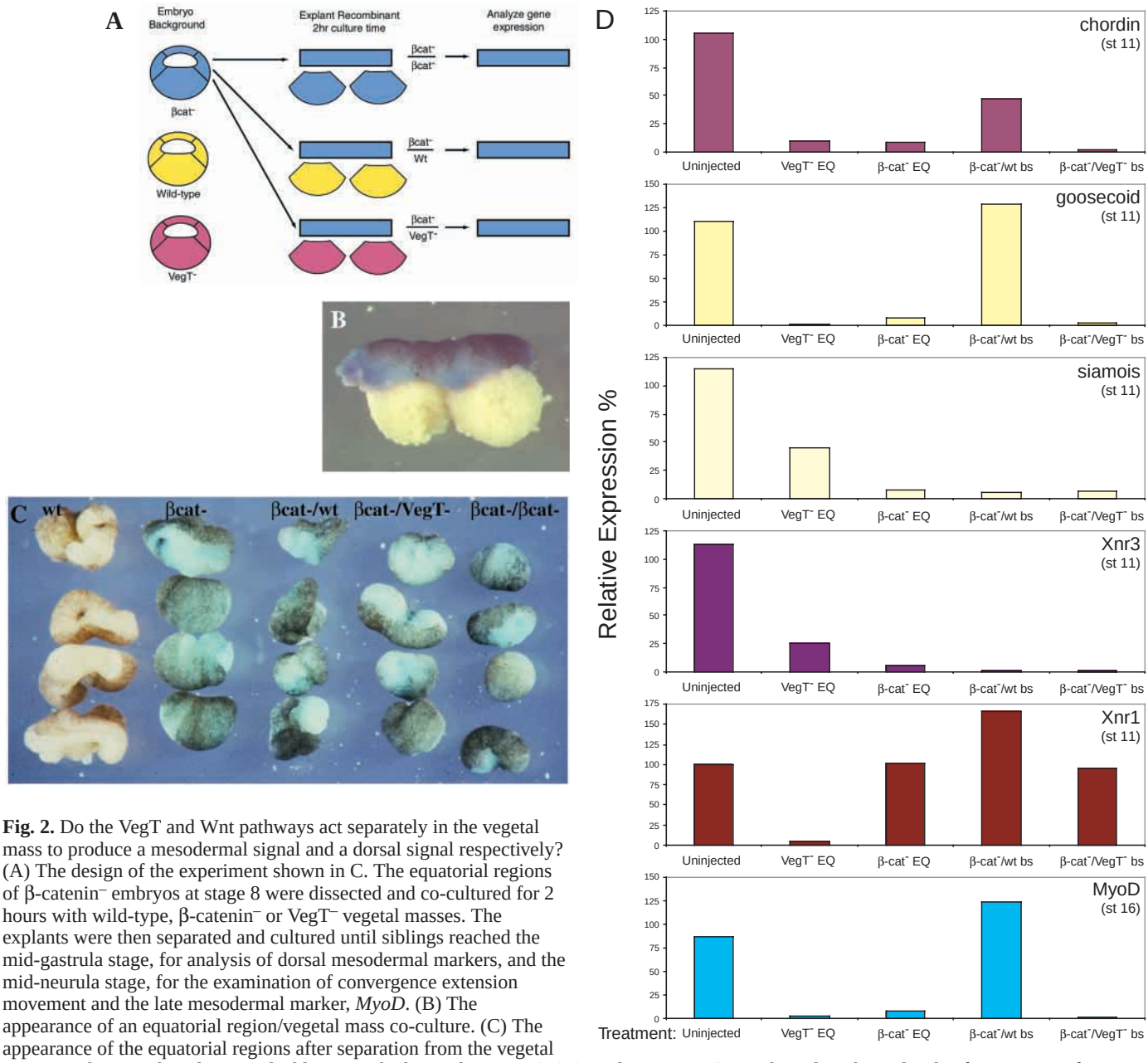


Fig. 2. Do the VegT and Wnt pathways act separately in the vegetal mass to produce a mesodermal signal and a dorsal signal respectively? (A) The design of the experiment shown in C. The equatorial regions of β-catenin⁻ embryos at stage 8 were dissected and co-cultured for 2 hours with wild-type, β-catenin⁻ or VegT⁻ vegetal masses. The explants were then separated and cultured until siblings reached the mid-gastrula stage, for analysis of dorsal mesodermal markers, and the mid-neurula stage, for the examination of convergence extension movement and the late mesodermal marker, *MyoD*. (B) The appearance of an equatorial region/vegetal mass co-culture. (C) The appearance of the equatorial regions after separation from the vegetal masses and overnight culture until siblings reached neurula stage 16. (D) Real-time RT-PCR to show the relative levels of expression of organizer genes in sibling equators of those shown in C and D, frozen at the gastrula stage (11, or 16 in last panel). EQ, equator; β-cat⁻/wt bs, β-catenin⁻ equator co-cultured with wild-type bases; β-cat⁻/VegT⁻ bs, β-catenin⁻ equator co-cultured with VegT⁻ bases. In each case, *ornithine decarboxylase* (ODC) is used as a loading control (data not shown), and each bar is normalized to the level of ODC.

VegT component in the vegetal mass, or could be supplied in the equatorial region, we tested the ability of β-catenin⁻ vegetal masses to rescue VegT⁻ equators (Fig. 3). Fig. 3 shows that the expression of *gsc* and *chordin* was partially rescued in this manner, indicating that Wnt signaling activity in the equator, together with inducing signals downstream of VegT in the vegetal mass, was sufficient to activate these genes. Both Figs 2 and 3 support the model of cell autonomous activity of Wnt signaling (derepressing XTcf-3 inhibition), combining with either direct VegT activation or indirect inductive activation via secreted signals downstream of VegT.

Each *Xnr* has a discrete dorsoventral pattern of expression over time; a pattern that is lost in β-catenin⁻ embryos

The patterning of mesoderm has been suggested to be the result of either a dorsoventral concentration gradient of Xnr activity (Agius et al., 2000), or the result of temporal differences in dorsoventral expression of the *Xnrs* (Lee et al., 2001). To study this further we examined the change in the amount and location of expression of *Xnr1*, *2*, *3* and *4* in β-catenin⁻ embryos over a 4-hour period during the onset of gastrulation.

We marked the dorsal side of wild-type and β-catenin⁻

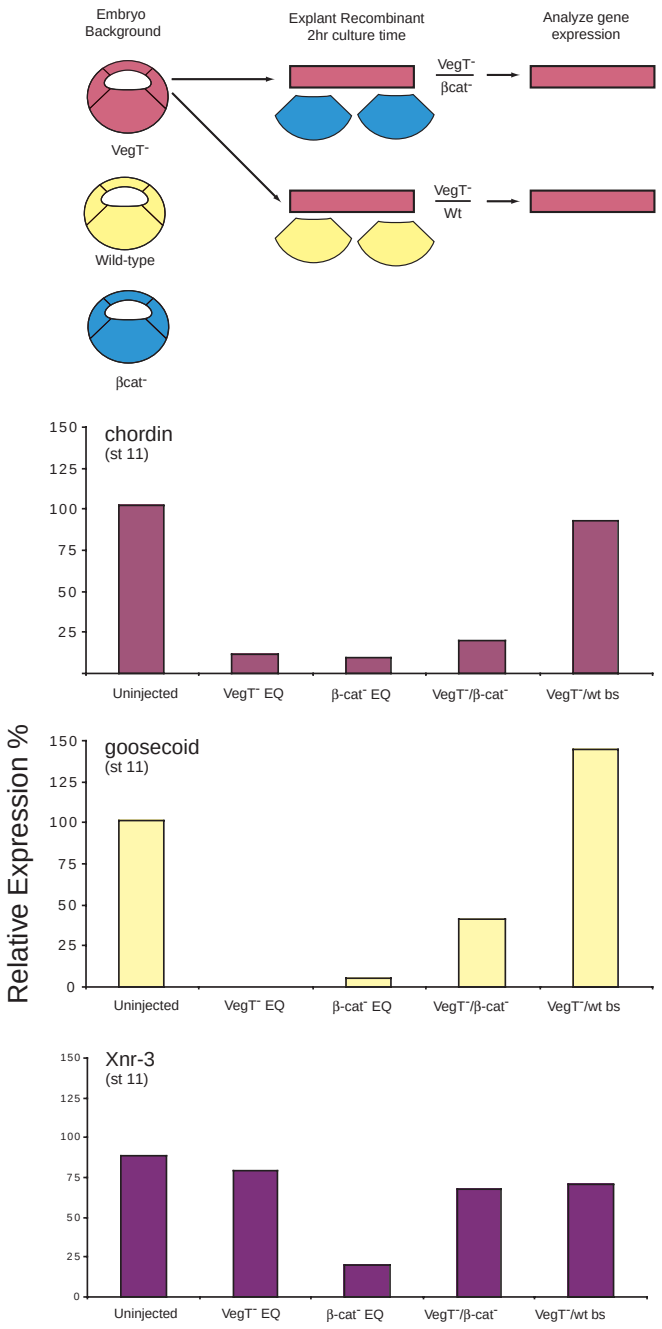


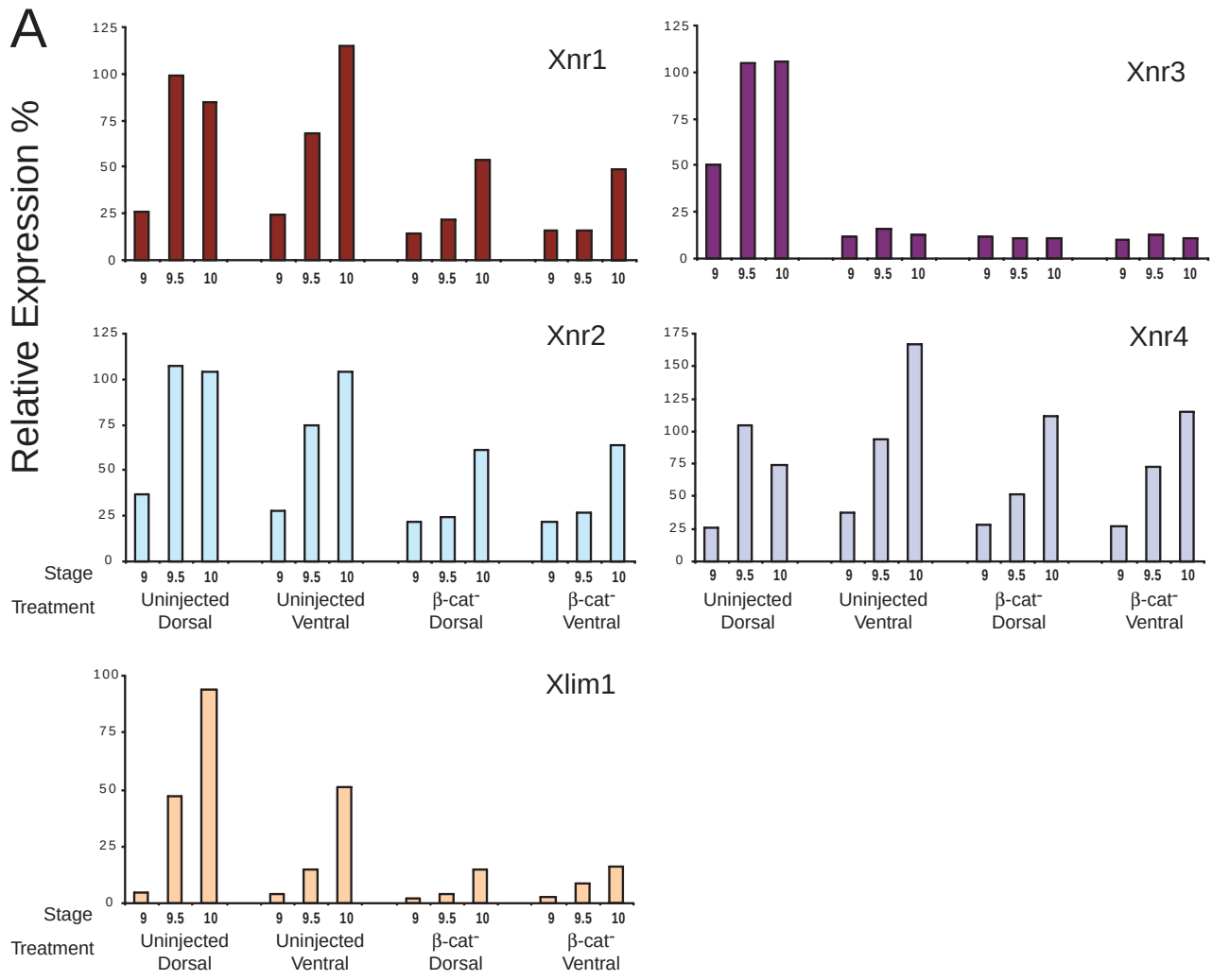
Fig. 3. The Wnt component controlling *goosecoid* and *chordin* expression can be supplied in the equatorial region while the VegT component comes from the vegetal mass. The experimental design is shown at the top. The histograms show the expression of *gsc* and *chordin* and *Xnr3* in isolated equatorial explants after culture with vegetal masses in the combinations shown. The expression of *gsc* and *chordin* was partially rescued in equatorial explants by co-culture with β-catenin⁻ vegetal masses, indicating that Wnt signaling activity in the equator together with inducing signals downstream of VegT in the vegetal mass are sufficient to activate these genes.

embryos with Nile Blue crystals at the eight-cell stage, and dissected them into dorsal and ventral halves at the mid-blastula stage. Half embryos were frozen in groups of four at stages 9, 9.5 and 10 and the expression of *Xnr1*, *2* and *4* was studied using real time RT-PCR (Fig. 4A). To show the accuracy of the dissections, we examined *Xnr3* expression, since it is known to be localized on the dorsal side of wild-type embryos during the early gastrula stage (Smith et al., 1995). Fig. 4A (top right) shows that, in control embryos, Xnr3 was concentrated in the dorsal half and its expression peaked at stage 9.5-10. In comparison, *Xnr1* and *2* were expressed both dorsally and ventrally in wild-type embryos, peaking first on the dorsal side at stage 9.5, followed by a similar peak on the ventral side at stage 10. In β-catenin⁻ embryos, the total expression of *Xnr1* and *2* was reduced by about 50% compared to peak control levels, and the dorsal to ventral temporal change of expression was lost. The pattern of expression was the same in both halves (Fig. 4A). Interestingly, *Xnr4* expression peaked dorsally in wild-type embryos at stage 9.5 and reached a 35% higher peak ventrally at stage 10. Again, the dorsoventral differences were lost in β-catenin⁻ embryos, even though these differences were more marked ventrally. This data is largely in agreement with previous work showing dorsal/ventral differences in the timing of expression of nodal genes (Agius et al., 2000; Lee et al., 2001). However, Lee et al. found that uv-ventralized embryos had maximal Smad2 phosphorylation levels comparable to those of wild-type embryos. This suggests that uv did not change the total amount of nodal signaling, although it was also possible that Smad2 phosphorylation might not be directly correlated with *Xnr* expression. Here we saw a clear reduction of nodal expression in β-catenin-depleted embryos.

To examine whether this pattern of expression of *Xnr1* mRNA was representative of other genes that are both dependent on VegT for their expression, and modulated by Wnt signaling, we also examined *Xlim1* mRNA (Fig. 4A). In wild-type embryos we confirmed it was also more abundant dorsally (Taira et al., 1993), and in β-catenin⁻ embryos the level of expression was reduced and symmetrical across the dorsal and ventral halves.

These experiments showed that β-catenin⁻ embryos express *Xnr1*, *2* and *4* but that their expression peaks at a later time than controls. We next asked whether vegetal masses from early gastrula stage β-catenin⁻ embryos (*Xnr*s now higher) could induce dorsal mesoderm in mid-blastula stage equators (Fig. 4B). We co-cultured equators from wild-type and β-catenin⁻ mid-blastula stage embryos with β-catenin⁻ vegetal masses from late blastulae (low *Xnr* expression) or early gastrula stage (*Xnr* now higher) for a 2-hour period. We have shown that vegetal masses of β-catenin⁻ embryos do not express *chordin*, *cerberus*, *noggin*, *crescent*, *Xnr3* or *Xnr6*, and this was also true for dissected vegetal masses (data not shown). Although *FGF*s were still expressed, we showed that their expression was in the equatorial region, not the vegetal mass (data not shown). The TGFβ, *derriere* was also still expressed in vegetal masses and could play a role in late inductions along with the *Xnr*s, since it was highly expressed at stage 10 (Fig. 1C).

The heterochronic co-cultures were then separated and the equators were cultured until siblings reached the late neurula stage (Fig. 4B). As shown in Fig. 4C, the dorsal mesodermal gene, *MyoD* was expressed in β-catenin⁻ equators that had been co-cultured for 2 hours with wild-type vegetal masses. In contrast, β-catenin⁻ blastula-stage vegetal masses did not



induce *MyoD* mRNA. However, β-catenin⁻ equators co-cultured with early gastrula-stage β-catenin⁻ vegetal masses did express *MyoD*. This evidence is consistent with the view that the early high expression of *Xnrs* is important for dorsal mesoderm induction. β-catenin depleted embryos might not form dorsal mesoderm because the early peak of *Xnr* expression was lost.

The importance of a Smad1 phosphorylation-free zone in axis formation

Next we studied the role of BMP inhibition in establishing the dorsal axis. To do this, we used a cleavage mutant form of BMP7, *cm-BMP7* mRNA (Hawley et al., 1995). First we showed that this blocks Smad1 phosphorylation at stage 10, when injected into one ventral vegetal blastomere of a wild-

Fig. 4. The timing, extent and localization of expression of Xnrs is regulated by the Wnt pathway and is necessary for inducing dorsal mesoderm in equatorial cells. (A) Real-time RT-PCR to show the relative levels of expression of the *nodal* genes *Xnr1, 2, 3* and *4* in dorsal and ventral halves of wild-type and β-catenin$^-$ embryos frozen at the late blastula and early gastrula stages (9, 9.5 and 10). The dorso-ventral pattern of expression of *Xnr1* seen in wild-type halves, is lost in β-catenin$^-$ embryos. ODC is used as a loading control (data not shown), and each bar is normalized to the level of ODC. (B) The design of the experiment shown in C. Equators from wild-type and β-catenin$^-$ mid-blastula stage embryos were co-cultured with with β-catenin$^-$ vegetal masses from mid-blastulae (β-cat$^-$/β-cat$^-$ early) or early gastrula stage (β-cat$^-$/β-cat$^-$ late). Controls were mid blastula β-catenin$^-$ equators co-cultured with wild-type mid-blastula vegetal masses (β-cat$^-$/WT). The co-cultures were then separated and equators were cultured until siblings reached the late neurula stage and they were then frozen for analysis. (C) Real-time RT-PCR to show the relative levels of expression of *MyoD* in equators from the experiment described in B. ODC is used as a loading control (data not shown), and each bar is normalized to the level of ODC.

type embryo at the eight-cell stage, without interfering with Smad2 phosphorylation (Fig. 5). Only one blastomere was injected since the aim here was to produce a Smad1 phosphorylation-free zone similar to that in the organizer (see Schohl and Fagotto, 2002), but not to block the BMP pathway across the entire embryo.

Since the Wnt and VegT pathway were both essential for the expression of organizer genes involved in antagonizing BMP signaling, we compared the extent to which inhibiting Smad1 phosphorylation in one area of β-catenin$^-$ and VegT$^-$ embryos rescued organizer and axis formation. One additional element to these experiments was to compare the effect of blocking BMP signaling on embryos lacking only maternal Wnt signaling (using a phosphorothioate oligo; m β-catenin$^-$), with the effect of blocking BMP signaling in embryos in which both maternal and zygotic β-catenin signaling was prevented using a morpholino oligo (m/z β-catenin$^-$) (Heasman et al., 2000).

We found that *cm-BMP7* mRNA had no rescuing effects on VegT$^-$ embryos apart from the rescue of very abnormal cement glands (Fig. 6B). In contrast, *cm-BMP7* caused a complete rescue of the trunk axis in both types of β-catenin$^-$ embryos. Maternal β-catenin$^-$ embryos injected with *cm-BMP7* mRNA were headless in all cases (total of 30/30 cases in three experiments). When both maternal and zygotic Wnt signaling was blocked, an abnormal anterior axis formed with enlarged cement glands and heads (39/39 cases in three experiments). We also injected a dose range of 300 pg, 600 pg and 1.2 ng of *cm-BMP7* mRNA. The m β-catenin$^-$ embryos were headless when treated in this way and looked similar at all doses, while increasing doses of *cm-BMP7* mRNA in the m/z β-catenin$^-$ background resulted in increasingly large cement glands and further shortened body axis. In no cases did the rescued embryos develop normal eyes, hearts and endodermal organs and they died at the swimming tadpole stage (data not shown).

Next we compared a dorsal versus a ventral injection site, injecting 600 pg of *cm-BMP7* mRNA either dorsally or ventrally at the eight-cell stage into m β-catenin$^-$ and m/z β-catenin$^-$ embryos and saw no difference in the degree of rescue of the axis (data not shown).

To examine whether the rescued notochords and neural structures were formed cell autonomously or by induction, we co-injected *cm-BMP7* mRNA together with *lacZ* mRNA into one ventral cell at the eight-cell stage, in both m β-catenin$^-$ and m/z β-catenin$^-$ embryos. Blue cells derived from the injected blastomeres were found mostly in the rescued notochord and neural structures, indicating that the injected cells cell-autonomously changed fate (Fig. 6C-E). Morpholino-injected embryos had expanded anterior, folded brain tissue (Fig. 6D). In both cases, the axes contained notochords and neural tubes in histological sections (Fig. 6E,F and data not shown). Somite formation was induced in these embryos in agreement with the findings of Mariani et al. (Mariani et al., 2001), and muscles were able to twitch. Cement glands that formed in both VegT$^-$ and β-catenin$^-$, *cm-BMP7* mRNA-injected embryos contained very few blue cells, suggesting that most of this tissue was induced (data not shown). We conclude that blocking BMP signaling is sufficient to rescue both neural and notochord axis formation but not normal anterior patterning of β-catenin$^-$ embryos.

Since β-catenin$^-$ embryos rescued with *cm-BMP7* mRNA had axial structures, we next asked whether organizer genes were expressed in these embryos, by analysing gene expression in a staged series through the gastrula and neurula stages. Fig. 7 shows that there was no rescue of organizer genes at the early gastrula stage. This was the case throughout the gastrula stages (data not shown). At the early neurula stage (stage 13) some organizer genes, particularly *chordin* and *cerberus* began to be expressed, although *siamois* and *Xnr3* were not. Neural markers *nrp1* and *NCAM* were also rescued in both m and m/z β-catenin$^-$ embryos by the injection of *cm-BMP7* mRNA (data not shown). VegT$^-$ embryos injected with *cm-BMP7* mRNA showed no rescue of *chordin* or *cerberus* at either gastrula or neurula stages. Similarly, VegT$^-$/β-catenin$^-$ embryos were not rescued by the expression of *cm-BMP7* mRNA, indicating that the late expression of *chordin* and *cerberus* is dependent on the activity of the VegT pathway.

Since *cm-BMP7* is expected only to block BMP activity in a localized fashion, one possible explanation for the lack of complete rescue of head formation was that it was not a sufficiently potent BMP antagonist. Therefore we repeated the experiment using a dose response of *noggin* mRNA (1, 10 and

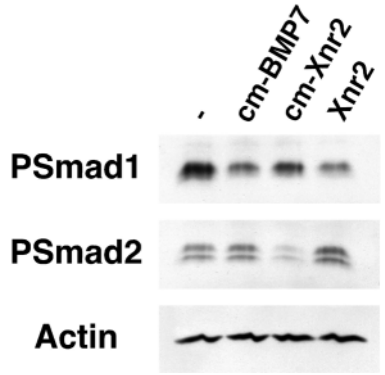


Fig. 5. *cm-BMP7* mRNA blocks Smad1 phosphorylation but not Smad2 phosphorylation. Wild-type embryos at the 8-cell stage were injected into one ventral blastomere with 600 pg of *cm-BMP7*, *cm-Xnr2* or *Xnr2* mRNAs. Embryos were cultured to stage 10 and frozen in groups of 5 for a western blot. Embryos were processed as described by Lee et al. (Lee et al., 2001). The blot was probed for both phosphorylated Smad1 and Smad2 protein. *cm-BMP7* mRNA-injected embryos had reduced expression of PSmad1 compared to uninjected controls, while PSmad2 was unaffected (lane 2). In comparison *cm-Xnr2* mRNA reduced Smad2 phosphorylation and increased PSmad1 (lane 3), while *Xnr2* mRNA increased PSmad2 and reduced PSmad1.

50 pg; Fig. 8). Fig. 8 shows that m/z β-catenin⁻ embryos rescued with *noggin* mRNA had similar degrees of axis formation to those rescued with *cm-BMP7* mRNA. Increasing the dose of *noggin* mRNA increased the size of the notochord and cement gland and shortened the body axis, but did not lead to normal head formation. Lower doses caused incomplete rescue of the dorsal axis. m β-catenin⁻ embryos rescued with *noggin* mRNA also phenocopied those rescued with *cm-BMP7* mRNA; they formed a normal dorsoventral axis but lacked heads (data not shown).

DISCUSSION

The results of this work and that of many others is summarized in a model (Fig. 9) that concentrates on the different categories of interactions between the VegT and Wnt pathways. Wild-type embryos expressed some early zygotic genes (represented here by *chordin*, *cerberus* and *noggin*) in a restricted area of the dorsal vegetal and equatorial region during the 12-hour period studied from MBT to the mid-gastrula stage. These genes were not expressed throughout this period in either VegT⁻ or β-catenin⁻ embryos.

A second group of key molecules (*Xnr1*, *2* and *4*) were activated by VegT in both dorsal and ventral vegetal cells but their peaks of expression differed dorsally and ventrally, both in terms of the amount of expression and the timing of expression. We concur with work of Whitman that there was a temporal wave of expression, not a static gradient from dorsal to ventral (Lee et al., 2001). For *Xnr4*, the ventral peak of expression was higher than the dorsal one. We found that in β-catenin⁻ embryos, these genes together with *Xlim1* and *Xhex* (data not shown) were still expressed but, at a reduced level, and, significantly, were now in a symmetrical fashion dorsally versus ventrally. This suggested that the Wnt pathway regulated these genes both dorsally and ventrally. Most studies have concentrated on the dorsal nuclear accumulation of β-catenin. However, our findings and the fact that nuclear β-catenin has been recently described in ventral nuclei at the blastula stage (Schohl and Fagotto, 2002), raised the question of whether the same (XTcf3) or different DNA binding partners of β-catenin regulate the ventral expression. It was also possible that the modulation of ventral expression levels of *Xnrs* by the Wnt pathway was indirect, and happened as a result of signals from elsewhere.

A third group of key molecules, *siamois* and *Xnr3* were direct targets of the Wnt pathway and were off in β-catenin⁻ embryos. The expression of these genes was not rescued by co-culture of wild-type vegetal masses with β-catenin⁻ equatorial regions, showing that they could not be induced by vegetal signals.

The fact that in uv-ventralized embryos, several key genes, including *siamois*, *Xnr3* and *cerberus*, continued to be expressed (Brannon and Kimelman, 1996; Cui et al., 1996; Darras et al., 1997), suggested that uv did not phenocopy β-catenin depletion. Uv-ventralization has also been used as evidence that the Wnt pathway regulates zygotic *BMP* expression (Fainsod et al., 1994). We showed here that *BMP4* mRNA was not up-regulated in β-catenin⁻ embryos throughout the 12-hour period studied. The same was true for *BMP7* mRNA (data not shown). In situ hybridization experiments are necessary to determine whether the different effects on BMP expression seen with uv treatment and β-catenin depletion

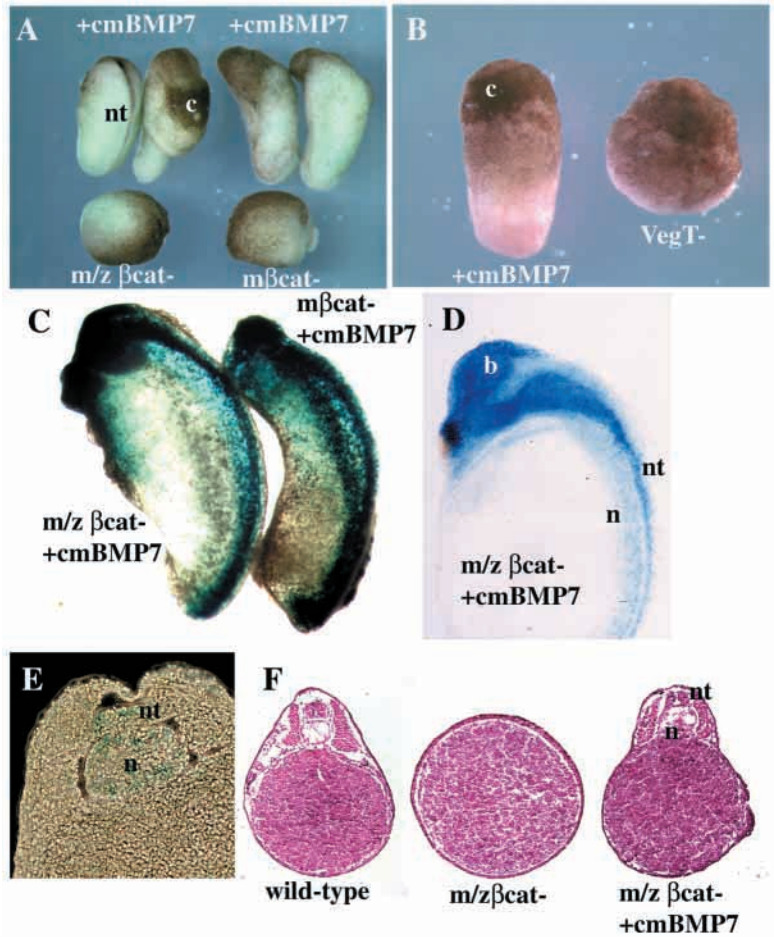


Fig. 6. The importance of a Smad1 phosphorylation- free zone in axis formation. (A) Embryos depleted of maternal (m βcat–), or both maternal and zygotic (m/z βcat–) β-catenin were injected with *cm-BMP7* mRNA (600 pg into 1 ventral vegetal cell at the 8-cell stage) and photographed at the tailbud stage. c, cement gland; nt, neural tube. (B) Embryos depleted of maternal *VegT* mRNA were injected with *cm-BMP7* mRNA (600 pg into 1 ventral vegetal cell at the 8-cell stage and photographed at the tailbud stage. c, cement gland. (C,D) *cm-BMP7* mRNA (600 pg) together with *lacZ* mRNA (200 pg) was injected into one ventral cell at the 8-cell stage, in both m β-catenin⁻ and m/z β-catenin⁻ embryos, that were then photographed at the tailbud stage. Blue cells derived from the injected blastomere were visible in cleared embryos mostly in the rescued notochord and neural structures. b, brain; n, notochord nt, neural tube. (E) Histological section of an m/z β-catenin⁻ embryo rescued by *cm-BMP7*/*Xga* mRNA injection. Note the blue cells in notochord (n) and neural tube (nt) in this phase contrast picture. (F) Hematoxylin and Eosin stained histological sections of wild-type, m/z β-catenin⁻ and m/z β-catenin⁻ +*cm-BMP7* mRNA injected embryos at the late tailbud stage. n,notochord; nt,neural tube.

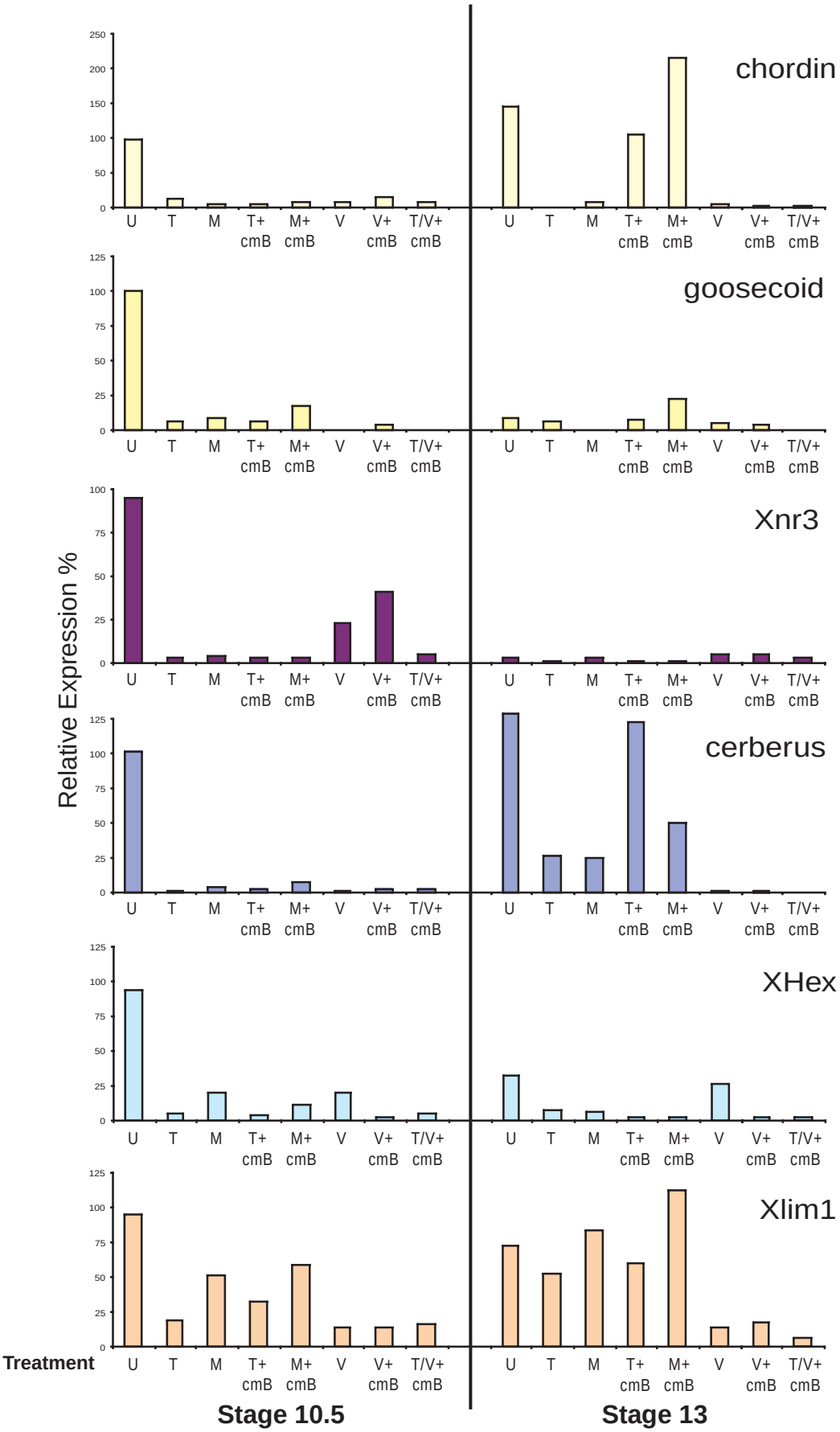


Fig. 7. Organizer gene expression is rescued at the early neurula stage by BMP blockade in β-catenin⁻ but not VegT⁻ embryos. Wild-type (uninjected, U), VegT⁻ thioate (V), m β-catenin⁻ thioate (T) and m/z β-catenin⁻ morpholino (M) embryos, and siblings injected with *cm-BMP7* (cmB) at the 8-cell stage (600 pg; 1 ventral vegetal cell) were frozen at the early gastrula (stage 10.5) and early neurula stage (stage 13) and analysed by real-time RT-PCR for organizer gene expression. The results are displayed as histograms to show the relative levels of expression. In each case, *ornithine decarboxylase* (ODC) is used as a loading control (data not shown), and each bar is normalized to the level of ODC expression.

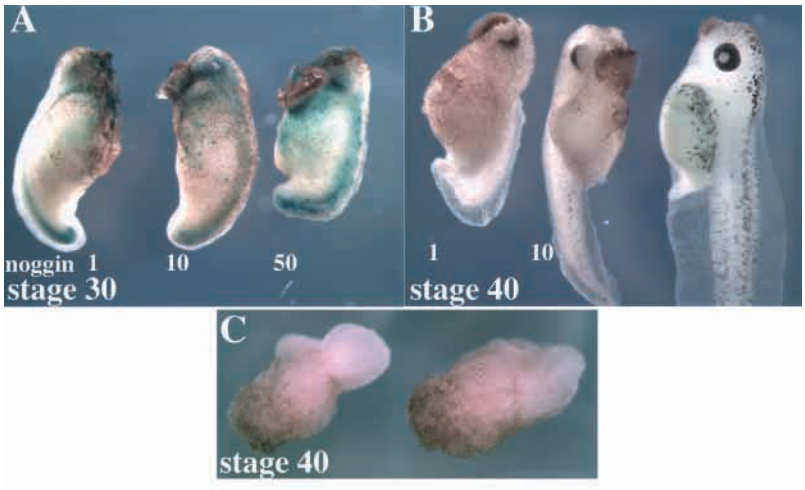


Fig. 8. Noggin also rescues normal dorsoventral but incomplete anterior axes in m/z β-catenin⁻ embryos. (A) Embryos depleted of both maternal and zygotic β-catenin (m/z β-catenin⁻)were injected with *noggin* mRNA (1, 10 or 50 pg) into 1 ventral vegetal cell a the 8-cell stage together with *lacZ* mRNA, and they were then processed for X-gal staining and photographed at the tailbud stage (stage 30). (B) Sibling embryos of those shown in A at stage 40. An uninjected control is included for comparison. Although the dorsoventral axis is normal, there are severe deficiencies in anterior patterning and axial length. (C) m/z β-catenin⁻ control embryos from this experiment.

experiments could be explained by redistribution of location of the mRNA.

A further difference between uv-treated and β-catenin⁻ embryos was in the degree to which BMP antagonists rescued axis formation. Studies have shown complete rescue of axes and normal heads in uv-irradiated embryos by *noggin* and *chordin* mRNA (e.g. Smith and Harland, 1992; Sasai et al., 1994). We showed here that normal head patterning was not rescued by similar doses of *noggin* mRNA in β-catenin⁻ embryos. Since β-catenin loss of function can be achieved easily by injecting β-catenin morpholino oligo into fertilized eggs, this provides a more reliable method to block Wnt pathway activity than using uv irradiation.

In this work we reinvestigated the question of a separate 'dorsal' signal in organizer formation. Previously, we have shown that wild-type vegetal masses induced both dorsal and general mesoderm in VegT⁻ equators, and that this induction was dependent on VegT, and Xnrs downstream of VegT (Kofron et al., 1999). Similarly, Agius et al., showed that animal caps could be induced to express dorsal, general and ventral mesodermal markers by co-culture with vegetal masses and this was blocked by an Xnr-blocking agent, Cer-S (Agius et al., 2000).

Dorsal signaling, as opposed to dorsal mesoderm induction, has been recognized as an entity in three contexts. A dorsalizing activity was demonstrated in the vegetal cytoplasm of oocytes and fertilized eggs by cytoplasmic transfer experiments (Holowacz and Elinson, 1995; Sakai, 1996; Darras et al., 1997; Marikawa and Elinson, 1999), and later in cleavage another dorsal inducer was recognized in dorsal vegetal cells, cells that have the ability to rescue axis formation when transplanted into uv-irradiated embryos, without changing their fate (Gimlich and Gerhart, 1984). That this activity was a Wnt pathway component was suggested by overexpression experiments using *Wnt* and *β-catenin* mRNA in animal caps (Christian et al., 1992; Wylie et al., 1996). Animal caps overexpressing β-catenin rescued *MyoD* expression in β-catenin⁻ equators (Wylie et al., 1996). However, the dorsalizing factor downstream of β-catenin remained unidentified (Marikawa and Elinson, 1999) and we questioned whether our over-expression experiments with β-catenin were reliable for reproducing endogenous dorsal signals. Transplantation experiments with dorsal vegetal cells of the 32-cell stage embryo also suggested that these cells acted as dorsal mesodermal inducers rather than dorsal signaling centers (Nagano et al., 2000).

Here, instead of using over-expression, we studied endogenous inductive signals coming from the vegetal mass at the late blastula stage. We used the equatorial region instead of animal caps since the equator forms the mesoderm and organizer of the embryo. We took advantage of the fact that we now know that mesoderm signals are downstream of VegT. If mesoderm induction is downstream of VegT, and dorsal signaling downstream of β-catenin, then a β-catenin⁻ equator (that forms mesoderm but not dorsal mesoderm) should be rescued by a VegT⁻ vegetal mass. We found that this was not the case. Our results here and in the accompanying paper are consistent with the interpretation that the Wnt pathway was acting with VegT, to de-repress XTcf3-inhibited genes in the equator and the vegetal mass, and to modulate the level and timing of *Xnr* expression.

Since the Wnt pathway has at least two roles, modulating *Xnr* expression across the vegetal hemisphere, and activating the expression of organizer genes dorsally, we wanted to understand the relative importance of these two functions for axis formation. To separate the role of Wnt pathway in modulating *Xnrs*, from its role in activating BMP inhibitors, we blocked BMP signaling in β-catenin⁻ embryos (to restore the effects of Chordin, Noggin and Cerberus) while maintaining the low Xnr/Smad2 activity. We used *cm-BMP7* mRNA to block BMP signaling. Previous over-expression studies showed co-immunoprecipitation of Flag-tagged cm-BMP7 and cm-nodal, suggesting that *cm-BMP7* might directly interact with Xnrs and therefore might block Smad2 as well as Smad1 phosphorylation in vivo (Yeo and Whitman, 2001). Here we showed that when 600 pg *cm-BMP7* mRNA was injected ventrally into eight-cell stage embryos it did not alter Smad2 phosphorylation, although it did reduce Smad1 phosphorylation significantly, indicating that cm-BMP7 was specific in the context used here.

By injecting *cm-BMP7* mRNA into one ventral blastomere at the eight-cell stage we mimicked the formation of a Smad1 phosphorylation-free zone in one area of the early gastrula (Fig. 9), and asked to what extent this rescued axis formation in VegT⁻ and β-catenin⁻ backgrounds. The differences between the VegT⁻ and β-catenin⁻ states were that VegT⁻ embryos lacked the expression of organizer, endodermal and

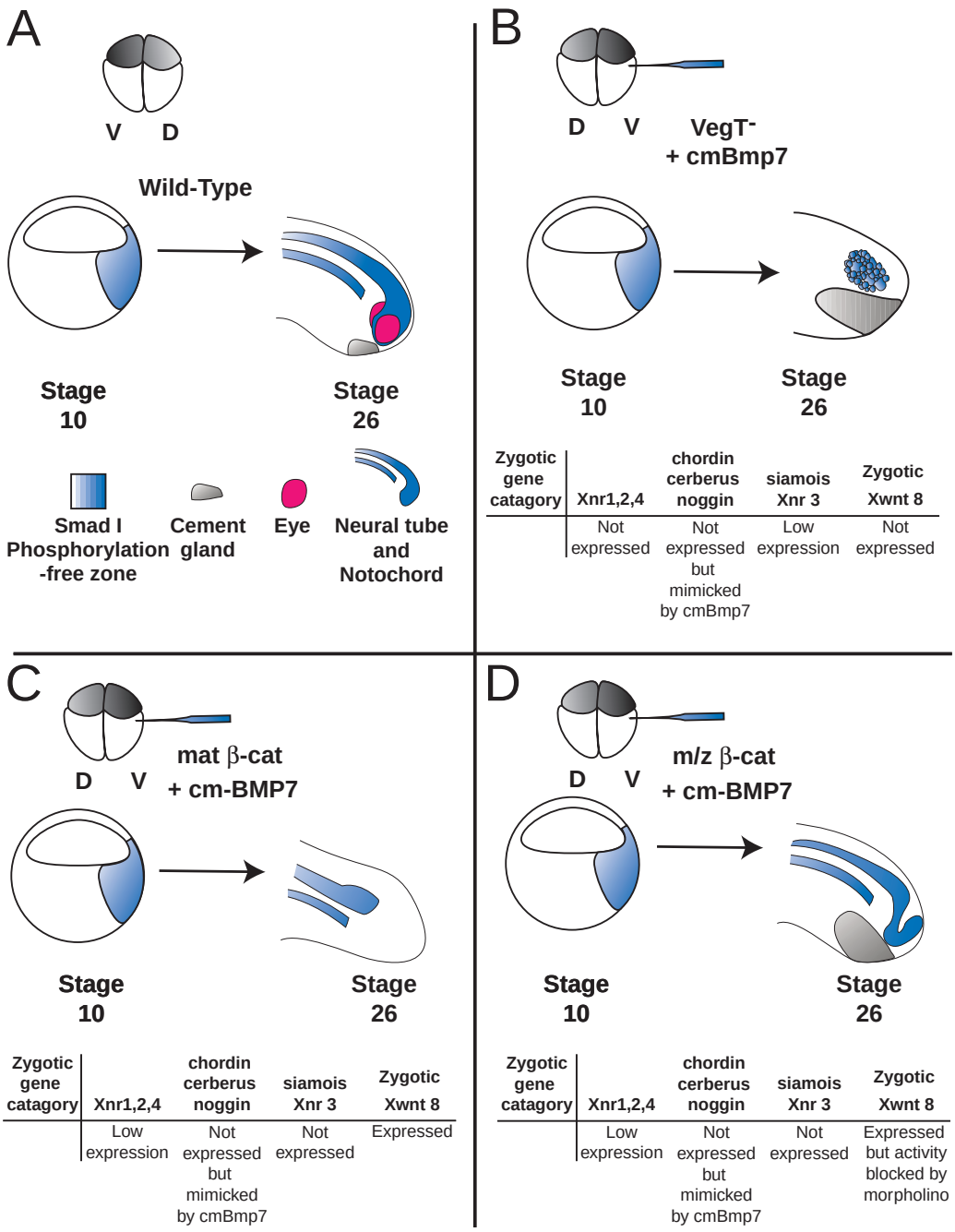


Fig. 9. Models of normal axis formation in wild-type embryos, compared to axes rescued in VegT⁻ and β-catenin⁻ embryos by the injection of *cm-BMP7* mRNA. (A) A diagrammatic view of normal axis formation at the gastrula (stage 10) and tailbud (stage 26) stages. For simplicity, the gastrulation movements that reverse the anterior-posterior axis are not drawn. (B) The lack of axis formation in a VegT⁻ embryo injected with *cm-BMP7* mRNA at the 8-cell stage in the ventral equatorial region, showing the gene expression profile of these embryos, throughout the gastrula stage. These embryos have abnormal cement glands but no neural tube or notochord. (C) The rescue of the trunk in m β-catenin⁻ injected embryos with *cm-BMP7* mRNA as in B. The progeny of the cells injected with *cm-BMP7* mRNA become the notochord and neural tube and induce somite formation. No cement gland or expanded brain forms because zygotic Wnt8 is expressed downstream of VegT and blocks anterior patterning. (D) The rescue of the trunk and partial rescue of the head in m/z β-catenin⁻ embryos injected with *cm-BMP7* mRNA as in B. They have notochords, abnormal brains, and enlarged cement glands. Zygotic *Wnt 8* mRNA is expressed but its function is blocked because the β-catenin morpholino oligo blocks translation of β-catenin protein. In neither C nor D do normal anterior structures form.

mesodermal genes, while β-catenin⁻ embryos had low, symmetrical *Xnr* expression, lacked the organizer genes *chordin*, *cerberus*, *noggin goosecoid*, *crescent*, *Xnr6*, *siamois* and *Xnr3*, expressed *derriere* and *FGF8*, and had low expression of *Xlim1* and *Xhex* (data not shown for *Xhex*). The rescue of VegT⁻ embryos was restricted to an enlarged cement gland. The fact that no axis was rescued here suggested that the genes involved in notochord/neural tube differentiation were downstream of VegT and thus could not simply rescued by blocking BMP signaling. Interestingly, we and others have shown that neural markers continued to be expressed in VegT⁻ embryos and *CerS* mRNA-injected embryos, even though they did not form recognizable neural tubes (Zhang et al., 1998; Wessely et al., 2001).

An important question here was to what extent, with a Smad1 inhibition area restored, would the β-catenin⁻ embryo be rescued. As illustrated in Fig. 6 the rescue was of a normal trunk axis but not of a normal head. This suggests that the elements still missing in the β-catenin⁻/*cm-BMP7* injected embryos were required for head patterning. While blocking zygotic β-catenin activity improved the anterior pattern by restoring a brain vesicle and enlarged cement gland, the forebrain did not form normally. Many other studies have indicated that zygotic *Wnt* inhibition is essential for anterior neural development (Baker et al., 1999; Erter et al., 2001; Hartley et al., 2001; Kiecker and Niehrs, 2001; Lekven et al., 2001; Yao and Kessler, 2001). In maternal β-catenin⁻ embryos, *Xwnt8* was overexpressed compared to controls (Fig. 1C),

offering an explanation for the headlessness when the Smad1 phosphorylation-free zone was restored.

As well as the abnormal anterior axis, *m/z β-catenin*$^{-}$ embryos injected with c*m-BMP7* mRNA had abnormal anterior endoderm and heart development and were not viable tadpoles. One likely missing component required for normal anterior patterning could be later BMP signaling, which would remain inhibited by the over-expression of *cm-BMP7* or *noggin* mRNAs. Also early organizer elements still missing in these embryos included the dorsoventral waves of *Xnr1*, *2* and *4* expression, the expression of *siamois*, *Xnr3* and the correct level of expression of *Xhex* and *Xlim1*. Many studies have implicated all of these in aspects of neural, head, heart and anterior endoderm specification (for review, see Niehrs, 1999). The challenge is to work out the hierarchy of the regulatory networks. One simple possibility is that the early high dorsal level of *Xnr* expression was needed for the high level of expression of *siamois*, *Xnr3*, *Xhex* and *Xlim1*. This view is supported by the fact that we showed previously that a dose response of *Xnr1, 2* and *4* mRNAs injected into VegT^{-} embryos restored increasing amounts of head formation (Kofron et al., 1999). However, the specific roles of individual Xnrs need yet to be examined by loss-of-function analysis.

How did the trunk axis develop with the correct dorsoventral pattern in the *cm-BMP7* rescue experiments? Cells in which BMP cleavage was inhibited presumably were themselves not receiving BMP signals and did not have phosphorylated Smad 1 activity. Their progeny differentiated as the majority of cells of the neural tube, brain and notochord (the blue cells in Fig. 6C,D and Fig. 8A), suggesting that a major role of BMP antagonists is in blocking autocrine or short range BMP signaling. Interestingly, even when the BMP antagonist was the secreted noggin protein, the blue secreting cells also predominantly became neural and notochord tissue, suggesting that noggin activity is short range. Yasuo and Lemaire also showed that cell autonomous notochord formation required co-repression of Smad1 and Wnt signaling, although they suggested that the transcriptional repressor *goosecoid* was required in adjacent tissue to maintain notochord differentiation (Yasuo and Lemaire, 2001). In our experiments *gsc* expression remained very low in the rescued embryos at the neurula stage indicating that it was not essential in this instance for notochord formation.

The conclusion from this work is that the trunk and head patterning mechanisms of the Spemann organizer are already set in the late blastula, and both are products of the interactions of the VegT and Wnt pathways. The BMP antagonists absolutely require both the Wnt and the VegT pathway for expression for trunk formation, while modulation of VegT target gene expression by the Wnt pathway is necessary for head formation. The above data and the data in the accompanying paper are consistent with a model where the role of the Wnt pathway in organizer formation is threefold and stepwise. Firstly, β-catenin derepresses XTcf3 repression of direct target genes such as *Xnr6*, *siamois* and *gsc*, and these are concomitantly activated by VegT or, in the case of *siamois* and *Xnr3*, by an unknown activator. Then the Wnt pathway enhances early dorsal *Xnr1*, *2* and *4* expression, which in turn activates gastrulation movements and enhances the expression of *chordin*, *cerberus*, *crescent*, *noggin* and other organizer genes. Next the Wnt pathway enhances the ventral level of *Xnr* expression, presumably to stimulate gastrulation movements and gene expression ventrally. One result of the second activity is the exclusion of BMP signaling activity from the dorsal area, since secreted chordin, noggin and cerberus bind BMPs. By the early gastrula stage (stage 10.25) this activity is evidenced by low Smad 1 phosphorylation in the organizer area and this zone is maintained throughout gastrulation (Faure et al., 2000; Schohl and Fagotto, 2002).

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REFERENCES

Agius, E., Oelgeschlager, M., Wessely, O., Kemp, C. and De Robertis, E. M. (2000). Endodermal Nodal-related signals and mesoderm induction in Xenopus. *Development* **127**, 1173-1183.

Baker, J. C., Beddington, R. S. and Harland, R. M. (1999). Wnt signaling in Xenopus embryos inhibits bmp4 expression and activates neural development. *Genes Dev.* **13**, 3149-3159.

Bouwmeester, T., Kim, S., Sasai, Y., Lu, B. and De Robertis, E. M. (1996). Cerberus is a head-inducing secreted factor expressed in the anterior endoderm of Spemann's organizer. *Nature* **382**, 595-601.

Brannon, M. and Kimelman, D. (1996). Activation of Siamois by the Wnt pathway. *Dev. Biol.* **180**, 344-347.

Brickman, J. M., Jones, C. M., Clements, M., Smith, J. C. and Beddington, R. S. (2000). Hex is a transcriptional repressor that contributes to anterior identity and suppresses Spemann organiser function. *Development* **127**, 2303-2315.

Carnac, G., Kodjabachian, L., Gurdon, J. B. and Lemaire, P. (1996). The homeobox gene Siamois is a target of the Wnt dorsalisation pathway and triggers organiser activity in the absence of mesoderm. *Development* **122**, 3055-3065.

Crease, D. J., Dyson, S. and Gurdon, J. B. (1998). Cooperation between the activin and Wnt pathways in the spatial control of organizer gene expression. *Proc. Natl. Acad. Sci. USA* **95**, 4398-4403.

Cui, Y., Tian, Q. and Christian, J. L. (1996). Synergistic effects of Vg1 and Wnt signals in the specification of dorsal mesoderm and endoderm. *Dev. Biol.* **180**, 22-34.

Dale, L., Howes, G., Price, B. M. J. and Smith, J. C. (1992). Bone morphogenetic protein 4:a ventralizing factor in early *Xenopus* development. *Development* **115**, 573-585.

Darras, S., Marikawa, Y., Elinson, R. P. and Lemaire, P. (1997). Animal and vegetal pole cells of early *Xenopus* embryos respond differently to maternal dorsal determinants: implications for the patterning of the organiser. *Development* **124**, 4275-4286.

Erter, C. E., Wilm, T. P., Basler, N., Wright, C. V. and Solnica-Krezel, L. (2001). Wnt8 is required in lateral mesendodermal precursors for neural posteriorization in vivo. *Development* **128**, 3571-3583.

Fainsod, A., Steinbeisser, H. and De Robertis, E. M. (1994). On the function of BMP-4 in patterning the marginal zone of the Xenopus embryo. *EMBO J.* **13**, 5015-5025.

Faure, S., Lee, M. A., Keller, T., ten Dijke, P. and Whitman, M. (2000). Endogenous patterns of TGFβ superfamily signaling during early Xenopus development. *Development* **127**, 2917-2931.

Friedle, H., Rastegar, S., Paul, H., Kaufmann, E. and Knochel, W. (1998). Xvent-1 mediates BMP-4-induced suppression of the dorsal-lip-specific early response gene XFD-1' in Xenopus embryos. *EMBO J.* **17**, 2298-3207.

Frisch, A. and Wright, C. V. (1998). XBMPRII, a novel *Xenopus* type II receptor mediating BMP signaling in embryonic tissues. *Development* **125**, 431-442.

Gimlich, R. L. and Gerhart, J. C. (1984). Early cellular interactions promote embryonic axis formation in Xenopus laevis. *Dev. Biol.* **104**, 117-130.

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

Hartley, K. O., Hardcastle, Z., Friday, R. V., Amaya, E. and Papalopulu, N. (2001). Transgenic Xenopus embryos reveal that anterior neural development requires continued suppression of BMP signaling after gastrulation. *Dev. Biol.* **238**, 168-184.

Hawley, S. H., Wunnenberg-Stapleton, K., Hashimoto, C., Laurent, M. N., Watabe, T., Blumberg, B. W. and Cho, K. W. (1995). Disruption of BMP

signals in embryonic Xenopus ectoderm leads to direct neural induction. *Genes Dev.* **9**, 2923-2935.

Heasman, J., Crawford, A., Goldstone, K., Garner-Hamrick, P., Gumbiner, B., McCrea, P., Kintner, C., Noro, C. Y. and Wylie, C. (1994). Overexpression of cadherins and underexpression of beta-catenin inhibit dorsal mesoderm induction in early Xenopus embryos. *Cell* **79**, 791-803.

Heasman, J., Kofron, M. and Wylie, C. (2000). Beta-catenin signaling activity dissected in the early Xenopus embryo: a novel antisense approach. *Dev. Biol.* **222**, 124-134.

Holowacz, T. and Elinson, R. P. (1995). Properties of the dorsal activity found in the vegetal cortical cytoplasm of *Xenopus* eggs. *Development* **121**, 2789-2798.

Hoppler, S. and Moon, R. T. (1998). BMP-2/-4 and Wnt-8 cooperatively pattern the Xenopus mesoderm. *Mech. Dev.* **71**, 119-129.

Ishikawa, T., Yoshioka, H., Ohuchi, H., Noji, S. and Nohno, T. (1995). Truncated type 11 receptor for BMP-4 induces secondary axial structures in *Xenopus* embryos. *Biochem. Biophys. Res. Commun.* **216**, 26-33.

Jones, C. M., Lyons, K. M., Lapan, P. M., Wright, C. V. E. and Hogan, B. M. L. (1992). DVR-4 (bone morphogenetic protein 4) as a posterior ventralizing factor in *Xenopus* mesoderm induction. *Development* **115**, 639-647.

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

Kiecker, C. and Niehrs, C. (2001). A morphogen gradient of Wnt/beta-catenin signalling regulates anteroposterior neural patterning in *Xenopus*. *Development* **128**, 4189-4201.

Kofron, M., Demel, T., Xanthos, J., Lohr, J., Sun, B., Sive, H., Osada, S., Wright, C., Wylie, C. and Heasman, J. (1999). Mesoderm induction in *Xenopus* is a zygotic event regulated by maternal VegT via TGFbeta growth factors. *Development* **126**, 5759-5770.

Laurent, M. N., Blitz, I. L., Hashimoto, C., Rothbacher, U. and Cho, K. W. (1997). The Xenopus homeobox gene twin mediates Wnt induction of goosecoid in establishment of Spemann's organizer. *Development* **124**, 4905-4916.

Lee, M. A., Heasman, J. and Whitman, M. (2001). Timing of endogenous activin-like signals and regional specification of the *Xenopus* embryo. *Development* **128**, 2939-2952.

Lekven, A. C., Thorpe, C. J., Waxman, J. S. and Moon, R. T. (2001). Zebrafish wnt8 encodes two wnt8 proteins on a bicistronic transcript and is required for mesoderm and neurectoderm patterning. *Dev. Cell* **1**, 103-114.

Mariani, F. V., Choi, G. B. and Harland, R. M. (2001). The neural plate specifies somite size in the Xenopus laevis gastrula. *Dev. Cell* **1**, 115-126.

Marikawa, Y. and Elinson, R. P. (1999). Relationship of vegetal cortical dorsal factors in the Xenopus egg with the Wnt/beta-catenin signaling pathway. *Mech. Dev.* **89**, 93-102.

Marom, K., Fainsod, A. and Steinbeisser, H. (1999). Patterning of the mesoderm involves several threshold responses to BMP-4 and Xwnt-8. *Mech. Dev*. **87**, 33-44.

Molenaar, M., Roose, J., Peterson, J., Venanzi, S., Clevers, H. and Destree, O. (1998). Differential expression of the HMG box transcription factors XTcf-3 and XLef-1 during early xenopus development. *Mech. Dev.* **75**, 151-154.

Moon, R. T. and Christian, J. L. (1992). Competence modifiers synergize with growth factors during mesoderm induction and patterning in *Xenopus*. *Cell* **71**, 709-712.

Nagano, T., Ito, Y., Tashiro, K., Kobayakawa, Y. and Sakai, M. (2000). Dorsal induction from dorsal vegetal cells in Xenopus occurs after mid-blastula transition. *Mech. Dev.* **93**, 3-14.

Niehrs, C. (1999). Head in the WNT: the molecular nature of Spemann's head organizer. *Trends Genet.* **15**, 314-319.

Nieuwkoop, P. D. (1969). The formation of mesoderm in Urodelean amphibians. I. Induction by the endoderm. *Wilhelm Roux' Arch. EntwMech. Org.* **162**, 341-373.

Nishita, M., Hashimoto, M. K., Ogata, S., Laurent, M. N., Ueno, N., Shibuya, H. and Cho, K. W. (2000). Interaction between Wnt and TGF-beta signalling pathways during formation of Spemann's organizer. *Nature* **403**, 781-785.

Osada, S. I. and Wright, C. V. (1999). Xenopus nodal-related signaling is essential for mesendodermal patterning during early embryogenesis. *Development* **126**, 3229-3240.

Sasai, Y., Lu, B., Steinbeisser, H., Geissert, D., Gont, L. K. and De Robertis, E. (1994). Xenopus chordin: a novel dorsalizing factor activated by organizer-specific homeobox genes. *Cell* **79**, 779-790.

Sakai, M. (1996). The vegetal determinants required for the Spemann organizer move equatorially during the first cell cycle. *Development* **122**, 2207-2214.

Schohl, A. and Fagotto, F. (2002). Beta-catenin, MAPK and Smad signaling during early *Xenopus* development. *Development* **129**, 37-52.

Smith, J. (1989). Mesoderm induction and mesoderm-inducing factors in early amphibian development. *Development* **105**, 665-677.

Smith, W. C. and Harland, R. M. (1992). Expression cloning of noggin, a new dorsalizing factor localized in the Spemann Organizer in Xenopus embryos. *Cell*, **70**, 829-840.

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

Steinbeisser, H., De Robertis, E. M., Ku, M., Kessler, D. S. and Melton, D. A. (1993). *Xenopus* axis formation: induction of goosecoid by injected Xwnt-8 and activin mRNAs. *Development* **118**, 499-507.

Steinbeisser, H., Fainsod, A., Niehrs, C., Sasai, Y. and De Robertis, E. M. (1995). The role of gsc and BMP-4 in dorsal-ventral patterning of the marginal zone in Xenopus: a loss-of-function study using antisense RNA. *EMBO J.* **14**, 5230-5243.

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

Taira, M., Otani, H., Good, P. and Dawid, I. (1993). The lim-domain containing homeobox gene *Xlim1* is expressed specifically in the organizer of *Xenopus* gastrula embryos. *Genes Dev.* **6**, 356-366.

Takahashi, S., Yokota, C., Takano, K., Tanegashima, K., Onuma, Y., Goto, J. and Asashima, M. (2000). Two novel nodal-related genes initiate early inductive events in Xenopus Nieuwkoop center. *Development* **127**, 5319-5329.

Tanegashima, K., Yokota, C., Takahashi, S. and Asashima, M. (2000). Expression cloning of Xantivin, a Xenopus lefty/antivin-related gene, involved in the regulation of activin signaling during mesoderm induction. *Mech. Dev.* **99**, 3-14.

Watabe, T., Kim, S., Candia, A., Rothbacher, U., Hashimoto, C., Inoue, K. and Cho, K. W. (1995). Molecular mechanisms of Spemann's organizer formation: conserved growth factor synergy between Xenopus and mouse. *Genes Dev.* **9**, 3038-3050.

Wessely, O., Agius, E., Oelgeschlager, M., Pera, E. M. and De Robertis, E. M. (2001). Neural induction in the absence of mesoderm: beta-catenin-dependent expression of secreted BMP antagonists at the blastula stage in Xenopus. *Dev. Biol.* **234**, 161-173.

Wylie, C., Kofron, M., Payne, C., Anderson, R., Hosobuchi, M., Joseph, E. and Heasman, J. (1996). Maternal beta-catenin establishes a 'dorsal signal' in early Xenopus embryos. *Development* **122**, 2987-2996.

Xanthos, J. B., Kofron, M., Wylie, C. and Heasman, J. (2001). Maternal VegT is the initiator of a molecular network specifying endoderm in Xenopus laevis. *Development* **128**, 167-180.

Yamamoto, T. S., Takagi, C., Hyodo, A. C. and Ueno, N. (2001). Suppression of head formation by Xmsx-1 through the inhibition of intracellular nodal signaling. *Development* **128**, 2769-2779.

Yao, J. and Kessler, D. S. (2001). Goosecoid promotes head organizer activity by direct repression of Xwnt8 in Spemann's organizer. *Development* **128**, 2975-2987.

Yasuo, H. and Lemaire, P. (2001). Role of Goosecoid, Xnot and Wnt antagonists in the maintenance of the notochord genetic programme in Xenopus gastrulae. *Development* **128**, 3783-3793.

Yeo, C. and Whitman, M. (2001). Nodal signals to Smads through Cripto-dependent and Cripto-independent mechanisms. *Mol. Cell* **7**, 949-957.

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

Zorn, A. M., Barish, G. D., Williams, B. O., Lavender, P., Klymkowsky, M. W. and Varmus, H. E. (1999a). Regulation of Wnt signaling by Sox proteins: XSox17 alpha/beta and XSox3 physically interact with beta-catenin. *Mol. Cell* **4**, 487-498.

Zorn, A. M., Butler, K. and Gurdon, J. B. (1999b). Anterior endomesoderm specification in Xenopus by Wnt/beta-catenin and TGF-beta signalling pathways. *Dev. Biol.* **209**, 282-297.

Zuck, M. V., Wylie, C. C. and Heasman, J. (1998). Maternal mRNAs in *Xenopus* embryos: an antisense approach. In *A Comparative Methods Approach to the Study of Oocytes and Embryos* (ed. J. D. Richter), pp. 341-354. Oxford: Oxford University Press.