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Repression of organizer genes in dorsal and ventral *Xenopus* cells mediated by maternal XTcf3

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SUMMARY

In the early *Xenopus* embryo, the dorsal axis is specified by a Wnt signal transduction pathway, involving the movement of β-catenin into dorsal cell nuclei and its functional association with the LEF-type transcription factor XTcf3. The subsequent function of XTcf3 is uncertain. Overexpression data has suggested that it can be both an activator and repressor of downstream genes. *XTcf3* mRNA is synthesized during oogenesis in *Xenopus* and is stored in the egg. To identify its role in dorsal axis specification, we depleted this maternal store in full-grown oocytes using antisense deoxyoligonucleotides, and fertilized them. The developmental effects of *XTcf3* depletion, both on morphogenesis and the expression of marker genes, show that primarily, XTcf3 is an inhibitor, preventing both dorsal and ventral cells of the late blastula from expressing dorsal genes. We also show that simple relief from the repression is not the only factor required for dorsal gene expression. To demonstrate this, we fertilized eggs that had been depleted of both *XTcf3* and the maternal transcription factor *VegT*. Dorsal genes normally repressed by XTcf3 are not activated in these embryos. These data show that normal dorsal gene expression in the embryo requires the transcriptional activator VegT, whilst XTcf3 prevents their inappropriate expression on the ventral side of the embryo.

Key words: *XTcf3*, Antisense oligo, *Xenopus*, Organizer, *VegT*

INTRODUCTION

The T cell factor (TCF) family of DNA binding proteins plays key roles in both embryonic development and in tumor progression. TCFs act as effectors of Wingless/Wnt signaling pathways by binding directly to armadillo/β-catenin (reviewed by Barker et al., 2000; Bienz, 1998; Nusse, 1999). They are known not to act as 'classical' transcription factors, but rather as components of multi-protein enhancer complexes (Hecht and Kemler, 2000). In *Xenopus* embryos, a maternal TCF family member, XTcf3, binds several co-activators and co-repressors including CBP (Hecht et al., 2000), Smad4 (Nishita et al., 2000) and groucho (Roose et al., 1998) as well as β-catenin (Molenaar et al., 1996). At this early stage of development, Wnt signaling is essential for the establishment of dorsoventral pattern. Embryos depleted of maternal *β-catenin* mRNA develop normally to the gastrula stage but then become radially symmetrical, lacking the dorsal elements of all three germ layers, including neural tubes, somites and notochords (Heasman et al., 1994). The precise role of XTcf3 in this process has not been resolved, but several models have been suggested.

First, XTcf3 could be a transcriptional activator of Wnt-inducible genes, such as *siamois* and *Xnr3*, when bound to β-catenin. This model was suggested by cell culture studies, where interaction of XTcf3 with β-catenin was required for the activation of a CAT reporter driven by a multimerized motif for TCF/LEF binding (CTTTGA/TA/T). XTcf3 alone had no activity (Molenaar et al., 1996). This model was also suggested by the fact that a truncated form of XTcf3, lacking the β-catenin-binding domain, repressed axis formation when expressed dorsally, and prevented the dorsalizing activity of ectopically expressed β-catenin (Molenaar et al., 1996). However, this construct could have acted in a dominant-negative fashion either for XTcf3 or for β-catenin itself, since it could block endogenous β-catenin activity. Second, XTcf3 has been suggested to act as a repressor ventrally, and as an activator dorsally in combination with β-catenin. In support of this, expression of a *siamois* promoter/CAT reporter containing mutated TCF-binding sites resulted in increased CAT expression when it was injected on the ventral side of a *Xenopus* blastula (Brannon et al., 1997).

No consistent picture has emerged from studies of the role of TCFs in other model systems. Loss-of-function studies in *Drosophila* support the view that a TCF/β-catenin complex activates *wingless* target genes, since *pangolin* (*dTCF*) mutants

phenocopy *wingless* and *armadillo* (*β-catenin*) mutant phenotypes (van de Wetering et al., 1997). However, in *C. elegans*, the *TCF* homologue, *pop1*, may be a transcriptional repressor, since *pop1* mutants have the opposite phenotype from *β-catenin* loss-of-function mutants (Rocheleau et al., 1997; Thorpe et al., 1997). Similarly, Tcf3 is considered to have a repressor function in zebrafish since the *headless* mutant phenotype, which maps to the *tcf3* gene, was rescued by the expression of a Tcf3-Engrailed repressor fusion protein (Kim et al., 2000).

In this situation, loss-of-function studies are essential. In *Xenopus*, zygotic transcription does not start until the mid-blastula stage, and *XTcf3*, like other early patterning genes, is expressed in the oocyte and stored as a maternal mRNA (Molenaar et al., 1998; Molenaar et al., 1996). We therefore specifically depleted this maternal store by microinjection of antisense deoxyoligonucleotides (oligos) against *XTcf3* into full-grown oocytes in culture, and fertilized them by the host-transfer technique. XTcf3$^-$ embryos developed with a dorso-anteriorized phenotype, and overexpressed the organizer genes *siamois*, *Xnr3*, *goosecoid* and *chordin*. These effects were specific to the depletion of *XTcf3* RNA, since two different oligos complementary to *XTcf3* mRNA had this effect and it was rescued by the reintroduction of *XTcf3* mRNA into oocytes. Organizer gene expression was increased on both the dorsal and ventral sides of XTcf3$^-$ embryos. *Siamois* and *Xnr3* expression was also activated in isolated animal caps. These results show that in vivo, XTcf3 acts as a repressor of organizer genes throughout the embryo. What then activates these genes in the organizer? The simplest model would be that β-catenin association with XTcf3 blocks the repression of dorsal gene transcription. Alternatively, there could be specific activators of dorsal gene transcription.

In a companion paper, we show that expression of many dorsal genes requires both the vegetally localized maternal transcriptional activator VegT and the maternal Wnt pathway (Xanthos et al., 2002). This suggests that VegT can activate organizer genes when β-catenin de-represses them. We tested this by depleting both *VegT* and *XTcf3* RNAs in the same embryos and confirmed that the dorsal markers *goosecoid* and *Xnr6* are regulated in this way.

MATERIALS AND METHODS

Oocytes and embryos

Oocytes were manually defolliculated and cultured as described previously (Kofron et al., 1999). Oocytes were injected with oligos in oocyte culture medium (OCM) using two equatorial injections per oocyte for XTcf3 oligos, or one vegetal injection for VegT and Axin oligos, cultured at 18°C and fertilized using the host transfer technique as described previously (Zuck et al., 1998). Rescue experiments were carried out as described in the text either by injecting mRNA equatorially into oocytes, 24 hours after the oligo (Fig. 2F), or by injecting into 4-cell stage embryos (Fig. 2E). Eggs were stripped and fertilized using a sperm suspension and embryos were maintained in 0.2× MMR. For injections of mRNA after fertilization (Fig. 2E), embryos were dejellied, and transferred to 2% Ficoll in 0.5× MMR at the 1-cell stage. mRNAs were diluted with sterile distilled water and injected into blastomeres.

For animal cap assays, mid-blastula embryos were placed on 2% agarose dishes in 1× MMR and the animal caps were dissected using sharp forceps. The caps were then cultured in OCM until sibling embryos reached the mid- to late-gastrula stage. For equatorial explants, embryos were placed on 2% agarose dishes at the mid-blastula stage and the equatorial regions were dissected with tungsten needles (Xanthos et al., 2001) and cultured in OCM until sibling embryos reached the mid-neurula stage. For separation into dorsal and ventral halves at the gastrula stage, 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 10, all the batches were placed on 2% agarose dishes in 1× MMR; pH 7.6 and bisected into dorsal and ventral halves, and frozen in groups of 4 half-embryos at 2-hour intervals through the gastrula stages.

Oligos and mRNAs

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

XTcf3 T1: 5′-C*G*A*G*GGATCCCAGTC*T*T*G*G-3′.

XTcf3 T2: 5′-G*A*G*ATAACTCTGA*T*G*G-3′.

VegT (VT9M): 5′-C*A*G*CAGCATGTACTT*G*G*C-3′ (Zhang et al., 1998).

Axin (M7): 5′-T*T*T*C*C*TCGCCAGGAA*C*T*G*G-3′ (Kofron et al., 2001).

The XTcf3 oligos are completely complementary to all four variants of XTcf3. Asterisks (*) represent phosphorothioate bonds. Oligos were resuspended in sterile, filtered water and injected in doses as described in the text. Full-length *XTcf3* in the vector pGlomyc was linearized with *Xba*I and capped *XTcf3* mRNA was synthesized using the T7 mMessage mMachine kit (Ambion). RNAs were phenol extracted, ethanol precipitated and then resuspended in sterile distilled water for injection.

Analysis of gene expression using real-time RT-PCR

Total RNA was prepared from oocytes, embryos and explants using proteinase K and then treated with RNase-free DNase as described previously (Zhang et al., 1998). Approximately one-sixth embryo equivalent of RNA was used for cDNA synthesis with oligo(dT) primers followed by real-time RT-PCR and quantitation using the LightCycler™ System (Roche) as described by Kofron et al. (Kofron et al., 2001). The primers and cycling conditions used are listed in Table 1. Relative expression values were calculated by comparison to a standard curve generated by serial dilution of uninjected control cDNA. Samples were normalized to levels of *ornithine decarboxylase* (ODC), which was used as a loading control. Samples of water alone or controls lacking reverse transcriptase in the cDNA synthesis reaction failed to give specific products in all cases.

RESULTS

Antisense oligos deplete *XTcf3* mRNA

We injected modified antisense chimeric phosphorothioate/phosphodiester deoxyoligonucleotides (oligos) into the equatorial regions of oocytes to deplete maternal *XTcf3* mRNA. RNA levels were assessed by real time RT-PCR. *XTcf3* mRNA was depleted to 30% of control levels by 3.5 ng of oligo T1 (Fig. 1). A second antisense oligo (T2) caused depletions to 10% control levels at 5 ng of oligo (Fig. 1B). Unexpectedly, we found that a second TCF, *XTcf4*, which had previously been reported to be first expressed at the neurula stage (Konig et al., 2000) was also expressed in the oocyte and early blastula stage embryo (Fig. 1A,B). Therefore we tested whether either of our *XTcf3*-specific oligos also depleted *XTcf4*. We found that T1 depleted *XTcf4* to 65% of control levels, while T2 was specific for *XTcf3*.

Table 1. PCR primer pairs and reaction conditions for real-time RT-PCR

PCR primer pair	Reference	Sequence	Denat. temp°C (2 sec)	Anneal temp°C/ time (sec)	Extension temp°C/ time (sec)	Acquisition temp°C/ time (sec)
Bmp4	New	U: 5′-ACCCATAGCTGCAAATGGAC-3′ D: 5′-CATGCTTCCCCTGATGAGTT-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
Goosecoid	New	U: 5′-TTCACCGATGAACAACTGGA-3′ D: 5′- TTCCACTTTTGGGCATTTTC-3′	95	55/5	72/11	82/3
IFABP	Shi and Hayes, 1994	U: 5′-GAAGGTCATAATCCAGCAAG-3′ D: 5′-CTGGTGAAAGTTCCAAGAAG-3′	95	60/5	72/11	81/3
Nkx2.3	New	U: 5′-TCGTGTATGTTGGCAGCAGGAG-3′ D: 5′-CCTCTTCATCTTCTTCTTTGGGGTC-3′	95	56/5	72/11	85/3
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
Plakoglobin	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
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
αT4 Globin	Kofron et al., 1999	U: 5′-AGC TGC CAA GCA CAT CGA-3′ D: 5′-GTG AGC TGT CCT TGC TGA-3′	95	56/5	72/12	86/3
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
Xlhbox8	Xanthos et al., 2001	U: 5′-GAA ATC CAC CAA ATC CCA CAC C-3′ D: 5′-TCC TTC TTC CAC TTC ATT CTC CG-3′	95	55/5	72/10	82/3
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′-TCCAGTATGATCCATCTGTTGC-3′ D: 5′-TTCTCGTTCCTCTTGTGCCTT-3′	95	55/5	72/10	83/3
Xsox17α	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
XTcf3	New	U:5′-ACG AAC ACT TCT CTC CAG GAA CCC-3′ D:5′-TGC CAC TAC CTT AGC CCT CAT CTC-3′	95	57/10	72/20	86/3
XTcf4	New	U: 5′-CTCCCCCATATCAGACTCCA-3′ D: 5′-GAGACGAGGGCAGAGAATTG-3′	95	60/5	72/9	86/3
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

Therefore we confirmed all of our findings with T1 using the T2 oligo. Depletion of maternal *XTcf3* did not affect the transcription of zygotic *XTcf3*, as its expression was equal to that of control embryos at the late gastrula stage (data not shown).

XTcf3-depleted embryos are dorso-anteriorized

Oocytes were matured in vitro and fertilized 36 hours after oligo injection. *XTcf3*-depleted (XTcf3^{-}) embryos developed normally through the cleavage and blastula stages, and became abnormal during gastrulation (Fig. 2A). High doses of oligo T1 (3.5 ng) caused delayed gastrulation, including a failure of blastopore closure, leading to abnormal development, including exogastrulation (Fig. 2B, red). Lower doses also slowed the appearance of the blastopore by 30 minutes, but gastrulation then proceeded (Fig. 2A). At the tailbud and tadpole stages, these embryos had dorso-anteriorized phenotypes (Fig. 2B, blue and mauve; 60/60 cases in 2 experiments). The notochord and anterior endoderm region were enlarged compared to controls, and the hindgut was concomitantly reduced. The most extreme cases of this phenotype at the swimming tadpole stage are shown in Fig. 2C. This experiment was repeated with the T2 oligo. 5 ng of this oligo caused the same effect as 3 ng of oligo T1 (Fig. 2F; 24/26 cases in 2 experiments).

We suspected that XTcf3^{-} embryos were dorsalized, and that excessive cell movements might be responsible for the gastrulation defects. To test this, we carried out explant assays on sibling embryos to those shown in Fig. 2B. Isolated equatorial regions of wild-type blastulae are known to undergo extensive convergence extension movements concomitant with notochord differentiation (Dale and Slack, 1987). We dissected the equatorial regions of wild-type and XTcf3^{-} embryos at the mid-blastula stage and cultured them in isolation until the mid-neurula stage. XTcf3^{-} explants underwent exaggerated and dose-dependent convergence extension movements compared to control explants (Fig. 2B, lower panel).

To confirm that this effect was specifically due to the

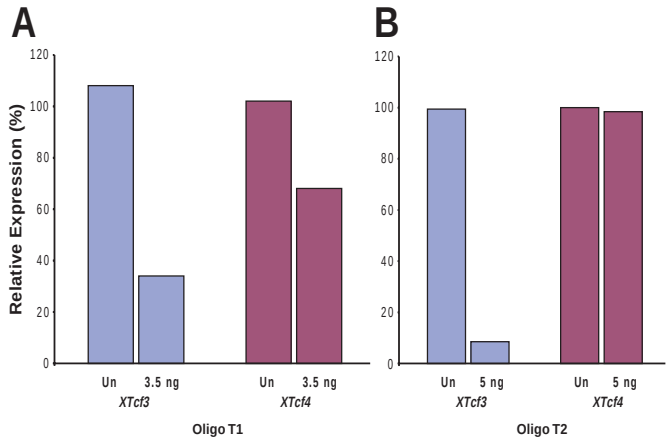


Fig. 1. Depletion of maternal *XTcf3* RNA. (A) Real-time PCR analysis of *XTcf3* and *XTcf4* RNA levels in uninjected oocytes (Un) and oocytes injected with 3.5 ng XTcf3 oligo T1. (B) Real-time PCR analysis of *XTcf3* and *XTcf4* RNA levels in uninjected oocytes (Un) and oocytes injected with 5 ng XTcf3 oligo T2.

depletion of *XTcf3*, we tested whether the dorso-anteriorized phenotype could be rescued by the introduction of synthetic *XTcf3* mRNA into XTcf3$^-$ oocytes. This experiment relies on the fact that both the targeted maternal mRNA and injected oligo degrade within 24 hours of injection. RNA injected after this time is not degraded by the oligo. XTcf3$^-$ oocytes were cultured for 36 hours and then half of them were injected equatorially with 100 pg of synthetic *XTcf3* mRNA either before (Fig. 2F) or after (Fig. 2E) fertilization. Embryos derived from these RNA-injected embryos were rescued (oligo T1, 12/43 cases dorso-anteriorized; oligo T2, 0/21 cases dorso-anteriorized) compared to those that received oligo only (oligo T1, 60/85 dorso-anteriorized oligo T2, 26/28 dorso-anteriorized) (Fig. 2E,F). In rescued embryos, the notochord was no longer protuberant, and the swollen anterior endoderm was reduced. In most cases the head was reduced in these embryos (Fig. 2E). Similar reduction in head structures was seen when 100-200 pg of *XTcf3* mRNA was injected into wild-type oocytes (not shown).

We next analyzed the levels of expression of early zygotic marker genes. XTcf3$^-$ and control embryos were frozen at 2-hourly intervals from the late blastula through the mid-gastrula stages, for real-time PCR analysis of a panel of organizer mRNAs. The organizer genes *siamois* and *Xnr3* have been shown to be direct targets of β-catenin/XTcf3 (Brannon et al., 1997; McKendry et al., 1997). Real-time RT-PCR analysis of XTcf3$^-$ gastrulae showed a dose-dependent increase in the expression of *siamois, Xnr3, goosecoid, chordin, cerberus* and *Xnr6* compared to control levels (Fig. 3A). Importantly, in the same experiment, all these markers, with the exception of *Xnr6*, were rescued or reduced to below wild-type levels by the introduction of *XTcf3* mRNA into XTcf3$^-$ oocytes (Fig. 3A). In comparison, the levels of *Bmp4* and *Xwnt8* were little affected in XTcf3$^-$ embryos compared to controls. This experiment was repeated using oligo T2 with the same result (data not shown).

In XTcf3$^-$ embryos at the tailbud stage, the ventral mesodermal marker *α-T4 globin* and general endoderm marker *IFABP* were expressed at reduced levels compared to controls, while the dorso-anterior endoderm marker *Xlhbox8* and heart marker *Nkx2.3* were increased (Fig. 3B). These experiments demonstrate that depletion of maternal *XTcf3* mRNA causes excessive dorso-anterior development.

XTcf3$^-$ embryos overexpress the direct target genes *siamois* and *Xnr3* both in the organizer and ectopically

XTcf3$^-$ embryos may develop with a dorso-anterior phenotype

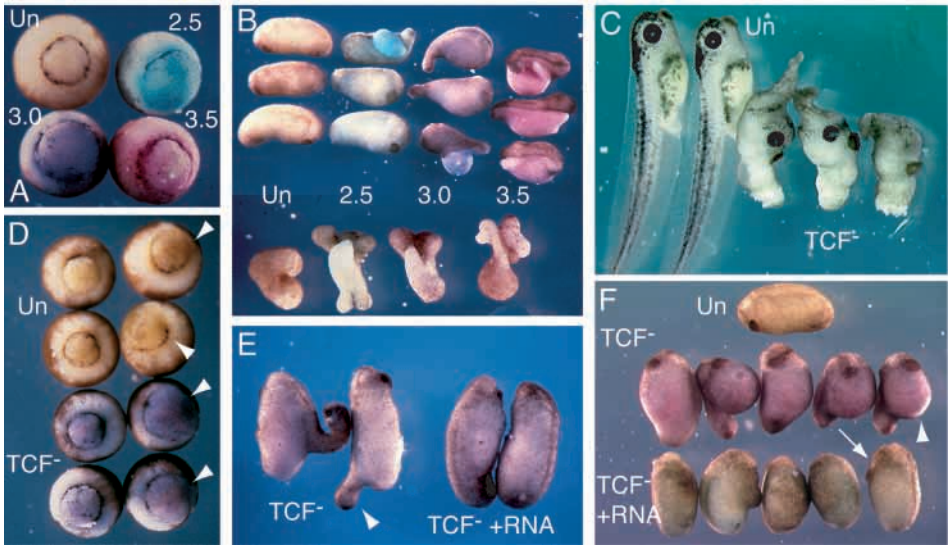


Fig. 2. Depletion of *XTcf3* causes a dorso-anteriorized phenotype. (A) Vegetal views of wild-type uninjected gastrula (top left; brown) and embryos injected with increasing doses of XTcf3 oligos (T1); 2.5 ng (top right; blue), 3.0 ng (bottom left; mauve) or 3.5 ng (bottom right; red). (B) Phenotypes of tailbud stage embryos (top) and isolated marginal zones (bottom) from uninjected controls (Un) or embryos injected with the indicated doses of XTcf3 oligos (T1; in ng). The embryos are from the same experiment as in A and the color code is the same. Excessive elongation is evident in isolated XTcf3$^-$ marginal zone explants. (C) Phenotype of XTcf3$^-$ embryos (TCF$^-$, 4 ng oligo T1) at the early tadpole stage (Un, uninjected). (D-F) Injection of *XTcf3* RNA can rescue the XTcf3$^-$ phenotype. These experiments used oligo T1 (3 ng, D,E) or T2 (5 ng, F). 100 pg of *XTcf3* RNA was injected in rescued embryos. (D)Vegetal views of uninjected (Un, brown) and XTcf3$^-$ gastrulae (TCF$^-$, mauve) compared to embryos from the same experiment injected with *XTcf3* RNA (right row, arrowheads). Note reduced blastopore protrusion and delayed gastrulation in the rescued XTcf3$^-$ embryos (bottom right two embryos). (E,F) Phenotypes of XTcf3$^-$ (TCF$^-$) and rescued embryos (TCF$^-$ +RNA) at the tailbud stage. Prominent notochords (arrowhead in E) and swollen anterior endoderm (arrowhead in F) in XTcf3$^-$ embryos are absent in rescued embryos. Heads are reduced in rescued embryos (arrows) owing to the ventralizing effect of overexpressed XTcf3.

because dorsal zygotic gene expression is increased by de-repression either in the organizer alone or throughout the embryo. To study this, we dissected explants from wild-type and XTcf3⁻ embryos and analyzed the expression levels of the organizer genes, *siamois*, *Xnr3*, *gsc* and *chordin* by real-time RT-PCR. First we isolated animal caps, which in isolation do not normally express dorsal markers, from XTcf3⁻ and uninjected control embryos at the blastula stage and assayed

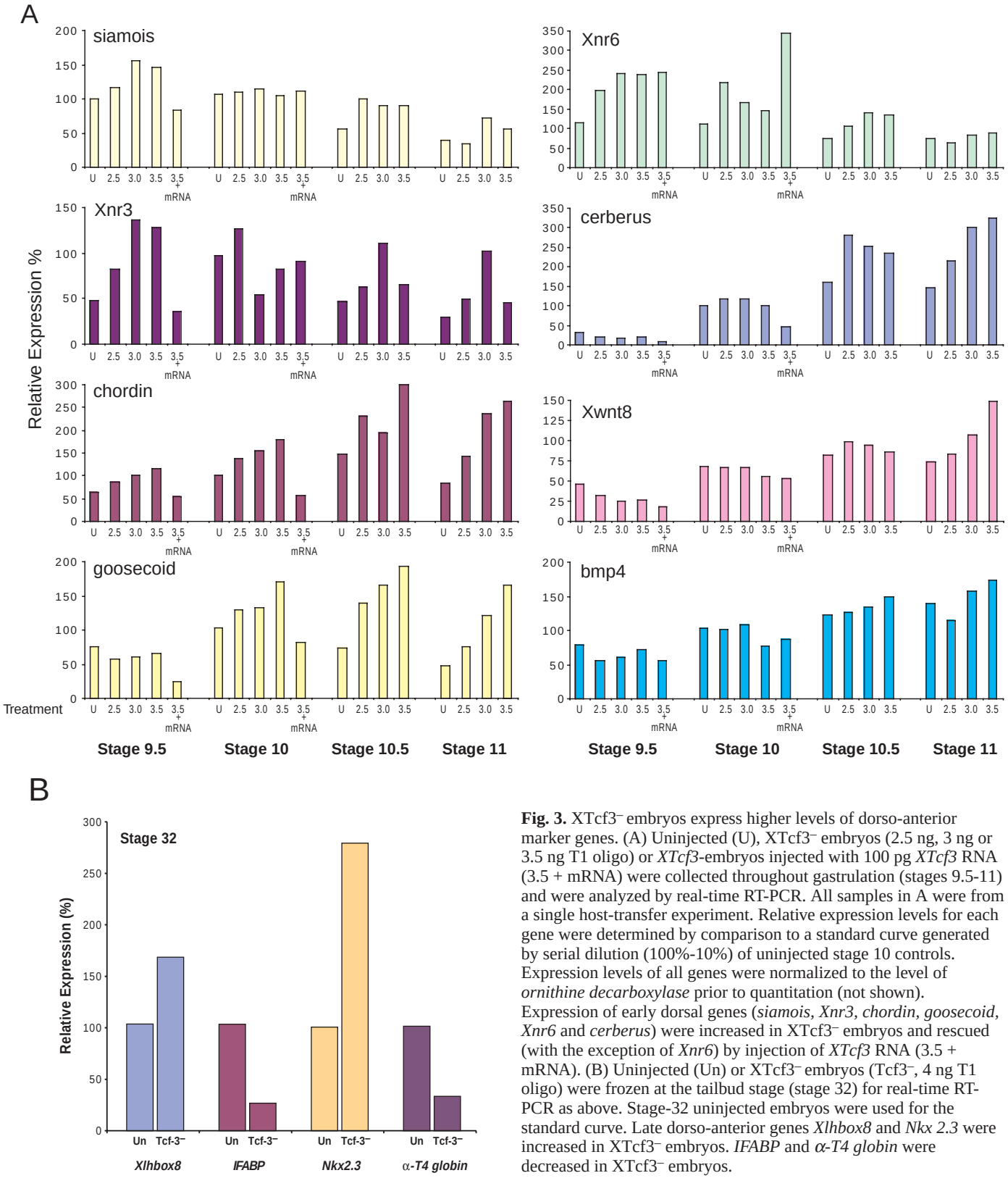


Fig. 3. XTcf3⁻ embryos express higher levels of dorso-anterior marker genes. (A) Uninjected (U), XTcf3⁻ embryos (2.5 ng, 3 ng or 3.5 ng T1 oligo) or *XTcf3*-embryos injected with 100 pg *XTcf3* RNA (3.5 + mRNA) were collected throughout gastrulation (stages 9.5-11) and were analyzed by real-time RT-PCR. All samples in A were from a single host-transfer experiment. Relative expression levels for each gene were determined by comparison to a standard curve generated by serial dilution (100%-10%) of uninjected stage 10 controls. Expression levels of all genes were normalized to the level of *ornithine decarboxylase* prior to quantitation (not shown). Expression of early dorsal genes (*siamois*, *Xnr3*, *chordin*, *goosecoid*, *Xnr6* and *cerberus*) were increased in XTcf3⁻ embryos and rescued (with the exception of *Xnr6*) by injection of *XTcf3* RNA (3.5 + mRNA). (B) Uninjected (Un) or XTcf3⁻ embryos (Tcf3⁻, 4 ng T1 oligo) were frozen at the tailbud stage (stage 32) for real-time RT-PCR as above. Stage-32 uninjected embryos were used for the standard curve. Late dorso-anterior genes *Xlhbox8* and *Nkx 2.3* were increased in XTcf3⁻ embryos. *IFABP* and *α-T4 globin* were decreased in XTcf3⁻ embryos.

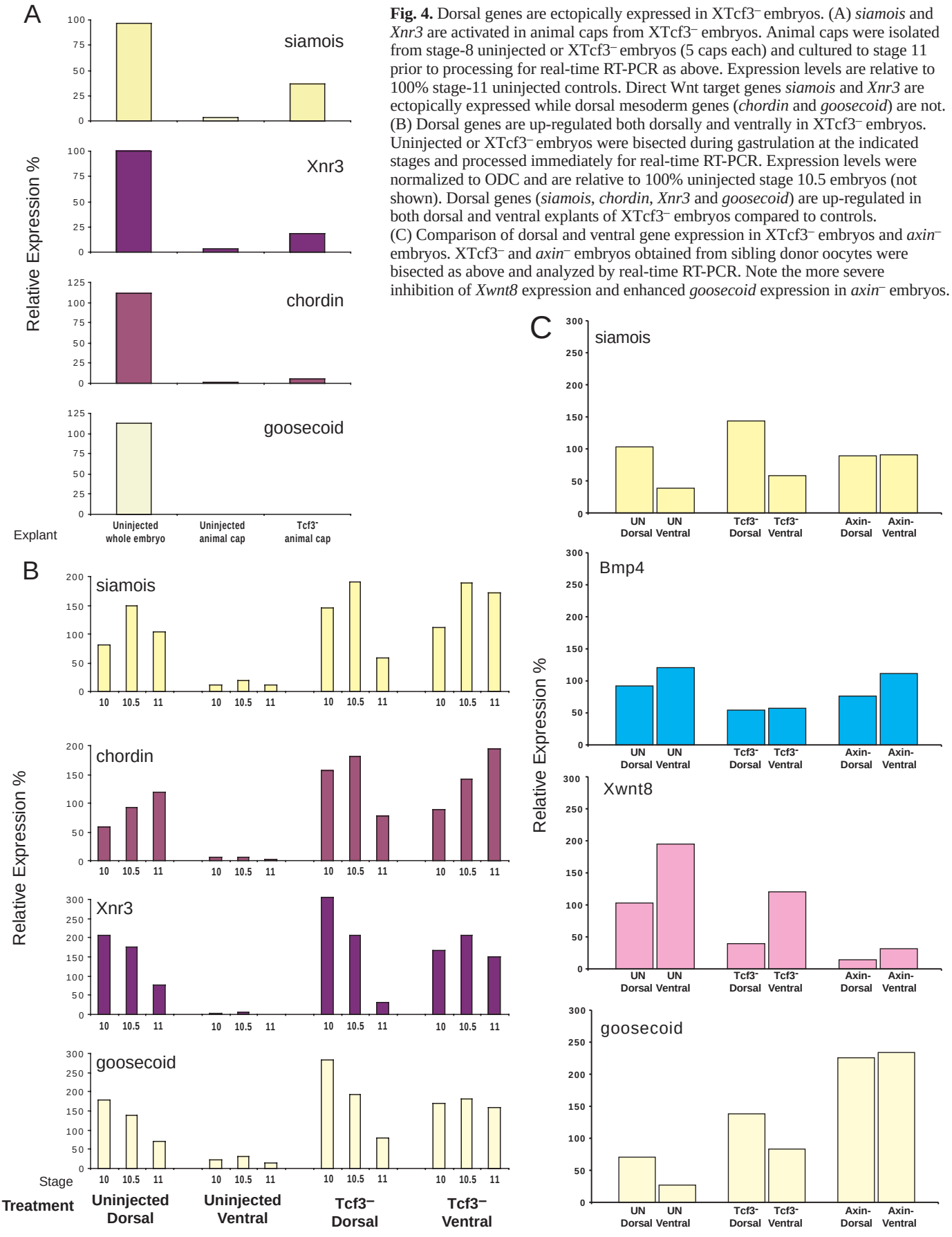


Fig. 4. Dorsal genes are ectopically expressed in XTcf3⁻ embryos. (A) *siamois* and *Xnr3* are activated in animal caps from XTcf3⁻ embryos. Animal caps were isolated from stage-8 uninjected or XTcf3⁻ embryos (5 caps each) and cultured to stage 11 prior to processing for real-time RT-PCR as above. Expression levels are relative to 100% stage-11 uninjected controls. Direct Wnt target genes *siamois* and *Xnr3* are ectopically expressed while dorsal mesoderm genes (*chordin* and *goosecoid*) are not. (B) Dorsal genes are up-regulated both dorsally and ventrally in XTcf3⁻ embryos. Uninjected or XTcf3⁻ embryos were bisected during gastrulation at the indicated stages and processed immediately for real-time RT-PCR. Expression levels were normalized to ODC and are relative to 100% uninjected stage 10.5 embryos (not shown). Dorsal genes (*siamois*, *chordin*, *Xnr3* and *goosecoid*) are up-regulated in both dorsal and ventral explants of XTcf3⁻ embryos compared to controls. (C) Comparison of dorsal and ventral gene expression in XTcf3⁻ embryos and *axin*⁻ embryos. XTcf3⁻ and *axin*⁻ embryos obtained from sibling donor oocytes were bisected as above and analyzed by real-time RT-PCR. Note the more severe inhibition of *Xwnt8* expression and enhanced *goosecoid* expression in *axin*⁻ embryos.

siamois, *Xnr3*, *gsc* and *chordin* expression during gastrulation. XTcf3$^-$ animal caps showed activation of *siamois* and *Xnr3* expression compared to control caps while *gsc* and *chordin* were not expressed (Fig. 4A). Next we examined the expression of *siamois*, *Xnr3*, *gsc* and *chordin* in dorsal and ventral halves of embryos bisected at three time-points during the gastrula stage (Fig. 4B). In control embryos at all three stages, more than 90% of the expression of *chordin*, *siamois* and *Xnr3* was restricted to the dorsal side, with the peak of expression of *siamois* and *Xnr3* occurring at stage 10, and the peak of expression of *chordin* occurring at stage 11. In comparison, XTcf3$^-$ embryos expressed all three organizer markers both dorsally and ventrally. On the dorsal side, the expression level of *siamois* and *Xnr3* was approximately 1.5-fold higher than control levels at stage 10, and for *chordin* was 3-fold that of control levels at stage 10. On the ventral side the expression of *siamois* and *Xnr3* was increased 15 fold at stage 10, while *chordin* levels peaked to the same extent at stage 10.5. In stage 10.5 XTcf3$^-$ embryos, the organizer genes were expressed equally highly on the dorsal and ventral sides (Fig. 4B). This experiment was repeated three times with the same result. These data show that the normal function of XTcf3 in vivo is transcriptional repression of the organizer genes *siamois*, *Xnr3* and *chordin* in the entire embryo throughout gastrulation.

Embryos dorsalized by *XTcf3* depletion do not phenocopy that of *axin* depletion

Axin is a major regulator of β-catenin degradation, and we have shown previously that its depletion results in both dorsal and ventral overexpression of β-catenin protein, and the overexpression of organizer genes both dorsally and ventrally (Kofron et al., 2001). However, *axin*-deficient embryos have much more pronounced dorso-anterior characteristics than *XTcf3*-depleted embryos, including circumferential cement glands, vertically orientated notochords and lack of tails. To compare these differences directly, we depleted both *axin* and *XTcf3* in the same experiment and compared the effects on the expression of dorsal and ventral marker genes at gastrulation (Fig. 4C). Both depletions caused an increase in dorsal markers *siamois* and *goosecoid*, although the extent of overexpression was variable. One significant difference between *XTcf3* and *axin* depletions was on the expression of *Xwnt8*. In *axin*$^-$ embryos, *Xwnt8* was severely reduced in its expression both dorsally and ventrally, while in XTcf3$^-$ embryos its expression was little affected ventrally and reduced to about 40% control levels dorsally (Fig. 4C) (Kofron et al., 2001). Since it has been shown in zebrafish that a *wnt8* mutant has an anteriorized nervous system (Erter et al., 2001), it is likely that in *axin*$^-$ embryos, loss of *Xwnt8* expression enhances the dorsalizing effect of the overexpression of organizer genes. This anteriorization would not be expected to be as extreme in XTcf3$^-$ embryos, where *Xwnt8* expression is little affected.

The removal of XTcf3 inhibitory activity needs to be coupled with activation by VegT for the expression of dorsal genes

In the absence of XTcf3, the organizer genes were robustly activated on both dorsal and ventral sides of the embryo. One likely candidate that might be responsible for this activation is the maternal transcription factor VegT. We next tested this hypothesis by comparing the expression of organizer genes in wild-type, *XTcf3*-depleted, *VegT*-depleted and *XTcf3/VegT*-depleted embryos. If VegT is required to activate organizer genes, then they should not be expressed in the double depleted embryos, even though they are robustly expressed in XTcf3$^-$ embryos.

A series of mid and late blastula and early gastrula stage embryos was analyzed by real-time RT-PCR. In this experiment, doses of VegT and XTcf3 oligos were sufficient to cause incomplete phenotypes (6 ng VegT and 3 ng T1; Fig. 5A), since the double depleted embryos with higher doses did not survive beyond the gastrula stage. At these doses, *VegT*$^-$ embryos showed extremely reduced levels of *gsc*, *chordin* and *Xsox17* compared to controls, while *siamois*, *Xnr3* and *Xnr6* were partially reduced (Fig. 5B). XTcf3$^-$ embryos had increased levels of all these markers compared to controls (Fig. 5B). In comparison, embryos depleted of both, *VegT*$^-$/XTcf3$^-$ embryos, had reduced levels of all the organizer genes compared to the control levels, indicating that, as well as derepression by XTcf3, these genes need activation by VegT for normal levels of expression. This difference was particularly striking for *Gsc* and *Xnr6*, which were almost completely off in double depleted embryos, suggesting that VegT is the only activator of these genes. *Xsox17* and *chordin* were reduced in expression compared to controls. For *Xnr3* and *siamois*, their expression was delayed but then rose to wild-type levels by the gastrula stage, suggesting that another transcriptional activator in addition to VegT modulates their early expression.

DISCUSSION

XTcf3 prevents the ectopic activation of dorsal zygotic genes

XTcf3 mRNA is expressed throughout the early *Xenopus* embryo (Molenaar et al., 1998). We confirm that it has roles as a transcriptional repressor, in dorsal, ventral and animal cells, suggesting a model for its activity as shown in Fig. 6. In ventral cells, it prevents the ectopic activation of organizer genes (*Xnr6*, *gsc*, *chordin*, *cerberus*, *Xhex*, *siamois* and *Xnr3*) while dorsally, binding of XTcf3 by β-catenin relieves this inhibition. However, relief of inhibition is not sufficient to activate organizer genes. In cells of the vegetal hemisphere VegT is an essential transcriptional activator of organizer genes including *Xnr6* and *gsc*. In animal cells, XTcf3 also blocks the expression of *siamois* and *Xnr3*. The fact that these genes are activated in this region when XTcf3 is depleted suggests that a transcriptional activator of these genes is also present, or that the basal trancriptional machinery is sufficient to maintain this level of expression. However *chordin* and *goosecoid* are not activated in animal cells when XTcf3 is depleted, presumably because they have no endogenous animally localized activator. These results confirm and extend previous studies which have shown that *siamois* and *Xnr3* can be induced in animal caps by overexpression of Wnt pathway components whereas *chordin* and *gsc* cannot (Brannon and Kimelman, 1996; Carnac et al., 1996; Fagotto et al., 1997; Smith et al., 1995; Steinbeisser et al., 1993; Watabe et al., 1995). Our experiments do not address whether any of these

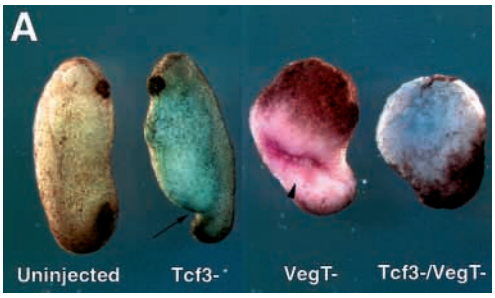
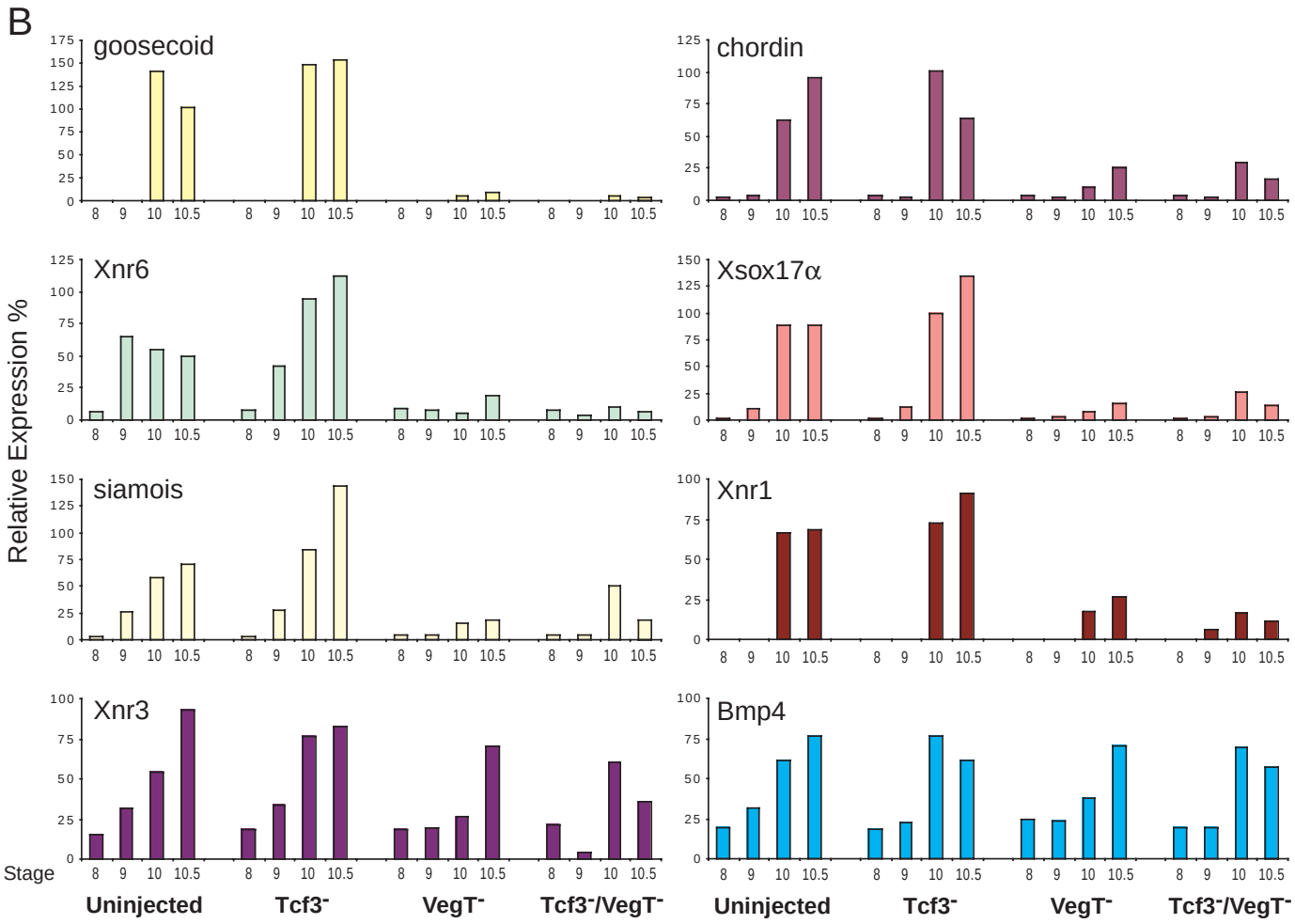


Fig. 5. Depletion of *XTcf3* does not rescue organizer formation in *VegT*– embryos. (A) Phenotypes of uninjected controls, XTcf3– (blue; 3 ng T1), *VegT*– (red; 6 ng) and XTcf3–/*VegT*– (mauve; 3 ng T1+6 ng VegT oligo) at the tailbud stage. XTcf3–/*VegT*– embryos resemble the *VegT*– embryo. (B) Real-time RT-PCR analysis of XTcf3–/*VegT*– embryos. Sibling embryos of those shown in A were collected at the indicated stages during gastrulation and analyzed for expression of dorsal/ventral patterning markers. *siamois* and *Xnr3* are up-regulated in XTcf3– embryos and remain at high levels in XTcf3–/*VegT*– embryos. *Goosecoid* and *Xnr6* are up-regulated in XTcf3– embryos but are not expressed in either *VegT*– or XTcf3–/*VegT*– embryos. *Chordin*, *Xnr1* and *Xsox17α* have a similar pattern of expression, but are not as severely affected as *goosecoid* and *Xnr6*. *Bmp4* is unaffected by depletion of either *XTcf3* or *VegT*.



regulatory mechanisms are direct or indirect interactions between the maternal signaling pathways and the zygotic target genes.

In the experiments presented here, we show a depletion rather than a complete loss of XTcf3 function, therefore we cannot discount the formal possibility that the residual amount of XTcf3 remaining may be sufficient to act as an activator of gene expression when it is present in small amounts. However, the dose response experiment (Fig. 3A) does not support a model where XTcf3 acts as a repressor at high concentrations and an activator at low concentrations, since increasing doses of oligo (causing graded depletion of *XTcf3* mRNA) causes an increasing expression of dorsal markers, not a biphasic response. Nor do we find evidence of genes that are simply activated by XTcf3 whose expression would be expected to be reduced by *XTcf3* depletion.

In the vegetal hemisphere, in XTcf3–/*VegT*– embryos, *gsc*, *Xnr1* and *Xnr6* have a very low level of expression, while *Xnr3* and *siamois* are still relatively robustly expressed. This suggests that *Xnr3* and *siamois* have an additional activating mechanism independent of VegT. Since *β-catenin*– embryos do not express *siamois* and *Xnr3*, it is likely that the unknown activator is also regulated by β-catenin. The suggestion that the Wnt pathway may regulate transcription factors other than XTcf3 in early *Xenopus* embryos was also raised in a recent study of the regulation of zygotic ventrolateral mesodermal genes, where β-catenin was shown to act independently of *XTcf3* (Hamilton et al., 2001). While further studies are needed

to identify the molecules involved, β-catenin has been shown to interact with other regulatory proteins including TCF4 (Korinek et al., 1997), Xsox17 and Xsox3 (Zorn et al., 1999) and retinoic acid receptor (RAR) (Easwaran et al., 1999).

Although organizer gene expression in XTcf3$^-$ embryos clearly shows that they are dorsalized, the embryos do not resemble *Xenopus* embryos dorsalized by the depletion of maternal *axin* (Kofron et al., 2001). Axin is a key component of the β-catenin degradation complex, and in its absence, organizer genes are also over-expressed both dorsally and ventrally (Kofron et al., 2001). However, *axin*-deficient embryos have much more pronounced dorso-anterior characteristics, including circumferential cement glands, vertically orientated notochords and lack of tails. There are several possible reasons for this. First, it is possible that the XTcf3$^-$ phenotype is very dose sensitive and we have not been able to pinpoint a dose between the dorsalized phenotype described in this work, and the arrest at gastrulation phenotype that would mimic the appearance of *axin*$^-$ embryos. Secondly, the XTcf3$^-$ phenotype may be qualitatively different from the *axin*$^-$ phenotype and not simply a less extreme form. We show that while *Xwnt8* is downregulated by *axin* depletion, it is less affected by *XTcf3* depletion (Fig. 3A, Fig. 4C). Since zygotic Wnts are known from other studies to be important in posteriorizing the axis (Erter et al., 2001; Kiecker and Niehrs, 2001; Xanthos et al., 2002), it is likely that this accounts for the more extreme anteriorization of *axin*$^-$ embryos. Additionally, *axin* depletion causes an increase in soluble β-catenin protein levels, whereas we found that *XTcf3* depletion had no effect on soluble β-catenin levels (data not shown). It is possible that β-catenin may activate or de-repress other DNA binding proteins as well as XTcf3, since XTcf3 is clearly not the only factor regulating *Xwnt8* in these experiments. Further experiments are needed to understand the regulators of zygotic *Xwnt8*.

The maternal Wnt pathway has also been implicated in regulating the zygotic expression of BMPs. This was first suggested in experiments where ventralization by ultraviolet irradiation caused an encroachment of *Bmp4* mRNA into the organizer region. Conversely, a dorsalizing lithium treatment resulted in a repression of *Bmp4* expression at the gastrula stage (Fainsod et al., 1994). Additionally, Baker et al. (Baker et al., 1999) showed that overexpression of Wnt pathway components can inhibit *Bmp4* expression during gastrulation. It is difficult to make comparisons between ventralizations and dorsalizations caused by ultraviolet radiation and lithium with those caused by loss-of-function of individual pathway components. However the results that we have from depletion of *β-catenin* (Xanthos et al., 2002), depletion of *axin* (Kofron et al., 2001) and depletion of *XTcf3* (Fig. 3A, Fig. 4C) are all consistent with the conclusion that there is little regulation of BMP expression by the Wnt pathway, at least until the midgastrula stage. This conclusion is supported by the fact that Schohl and Fagotto (Schohl and Fagotto, 2002) show in a

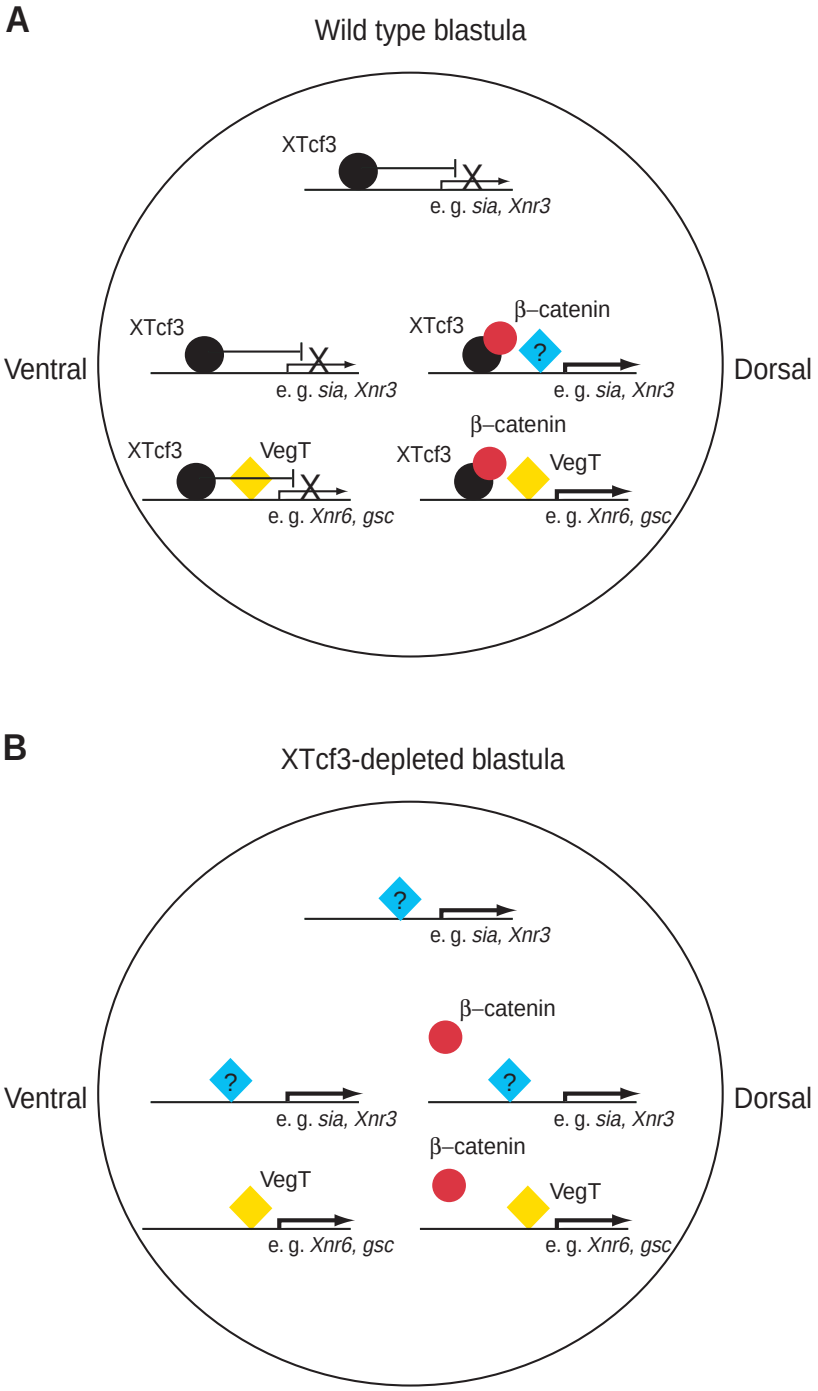


Fig. 6. Model for the roles of XTcf3 in regulating dorsal gene expression in the *Xenopus* blastula and gastula; XTcf3 acts solely as a repressor of organizer genes. (A) In the wild-type embryo, XTcf3 represses Wnt targets on the ventral side and in animal cap cells. Repression is alleviated dorsally by the action of stabilized β-catenin. VegT is also required for activation of *goosecoid* (*gsc*) and *Xnr6*. (B) In the absence of XTcf3, *siamois* (*sia*) and *Xnr3* are activated dorsally, ventrally and in animal cells via unknown activators. Genes such as *gsc* and *Xnr6* still require *VegT* for their activation.

recent paper that regions of Smad1 phosphorylation and nuclear β-catenin do not appear in a simple complementary pattern. An important question that remains to be answered is what does regulate zygotic BMP expression.

The results presented here are consistent with work in *C. elegans*, where the TCF homologue, *pop1*, was also found to be a transcriptional repressor (Rocheleau et al., 1997; Thorpe et al., 1997) and in zebrafish, where a Tcf3 homolog is thought to have a repressor function (Kim et al., 2000). However the phenotype of *Xenopus* XTcf3^{-} embryos is very different from the zebrafish *headless* mutant. One possible explanation for this difference might be that the zebrafish mutant may lack zygotic, but not maternal, Tcf3 function. Since zygotic Wnt signaling causes posteriorization of axial structures (Erter et al., 2001; Kiecker and Niehrs, 2001; Xanthos et al., 2002), a mutant that lacks the Wnt inhibitory action of Tcf3 would be expected to have excessive Wnt signaling and therefore headlessness. Preliminary experiments with a morpholino oligo suggest that blocking *Xenopus* zygotic *XTcf3* also causes a headless phenotype.

The conclusion from this work is that maternally encoded XTcf3 normally represses the activation of dorsal (organizer) genes throughout the embryo during the late blastula/early gastrula stages in *Xenopus*. This is consistent with its global expression at these early stages. In addition, activation of the Wnt pathway, leading to relief of this repression, is not sufficient to activate all organizer genes. Additional specific activators are required; one of these is VegT.

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