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Maternal XTcf1 and XTcf4 have distinct roles in regulating Wnt target genes

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Abstract

Wnt signaling pathways have essential roles in developing embryos and adult tissue, and alterations in their function are implicated in many disease processes including cancers. The major nuclear transducers of Wnt signals are the Tcf/LEF family of transcription factors, which have binding sites for both the transcriptional co-repressor groucho, and the co-activator β-catenin. The early *Xenopus* embryo expresses three maternally inherited Tcf/LEF mRNAs, and their relative roles in regulating the expression of Wnt target genes are not understood. We have addressed this by using antisense oligonucleotides to deplete maternal *XTcf1* and *XTcf4* mRNAs in oocytes. We find that XTcf1 represses expression of Wnt target genes ventrally and laterally, and activates their expression dorsally. Double depletions of XTcf1 and XTcf3 suggest that they act cooperatively to repress Wnt target genes ventrally. In contrast, XTcf4 has no repressive role but is required to activate expression of *Xnr3* and *chordin* in organizer cells at the gastrula stage. This work provides evidence for distinct roles for XTcfs in regulating Wnt target gene expression.

Keywords: *Xenopus*; XTcf; Antisense; Wnt; *Chordin*; *Siamois*; *Xnr3*

Introduction

Lymphoid enhancer factor/T-cell factor (Lef/Tcf) transcription factors are considered to be molecular switches, acting as transcriptional repressors in embryonic and adult cells when groucho and/or CtBP is bound (Brannon et al., 1999; Molenaar et al., 2000), and as activators when Wnt signal transduction leads to the binding of β-catenin and the displacement of groucho (Brannon et al., 1997; Daniels and Weis, 2005; Hurlstone and Clevers, 2002). β-catenin/Tcf causes chromatin remodeling involving the interacting partners of β-catenin, including CBP/p300 and Brg1, and the activation of Wnt target genes (Barker et al., 2001; Hecht et al., 2000; Miyagishi et al., 2000; Takemaru and Moon, 2000). Early *Xenopus* embryos contain a complex array of XTcfs. Three XTcf family members are inherited by fertilized eggs as maternal transcripts, and both XTcf1 and XTcf4 are represented by more than one isoform, as a result of alternative splicing (Pukrop et al., 2001; Roel et al., 2002; Roel et al., 2003). Since all these Tcf protein forms bind both β-catenin and groucho, and all are ubiquitously expressed in the early *Xenopus* embryo, an important question is whether they have individual roles or act cooperatively in regulating Wnt target genes.

In *Xenopus*, the maternal Wnt11/β-catenin/Tcf pathway is essential for dorsal axis specification (Heasman et al., 1994; Tao et al., 2005). Two organizer genes, the homeobox transcription factor *siamois*/*twin* and the nodal related gene *Xnr3*, are direct targets of the maternal Wnt signaling pathway (Brannon et al., 1997; Fan et al., 1998; McKendry et al., 1997). XTcf3 is a β-catenin binding transcription factor and regulator of *Xnr3* and *siamois* transcription (Behrens et al., 1996; Molenaar et al., 1996). Ablation of the mouse homolog, Tcf3, causes ectopic up-regulation of target genes including *Foxa2* (Merrill et al., 2004). The bimodal activity of XTcf3 was suggested by the fact that, on the one hand, it binds the transcriptional co-repressor groucho (Molenaar et al., 2000; Roose et al., 1998), and on the other, that reporters driven by the *engrailed* or *siamois* promoters containing Tcf/LEF binding sites are activated by Wnt signaling (Brannon et al., 1997; McGrew et al., 1999). The 5′ regulatory sequence of *siamois* was shown to contain three Tcf/LEF binding sites, one of which was an inhibitory motif (Fan et al., 1998). Ablation of mouse Tcf3 also leads to ectopic up-regulation of some target genes and down-regulation of others (Merrill et al., 2004). However, regulation of *siamois* and *Xnr3* cannot simply be

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explained by the bimodal function of XTcf3, since abrogation of XTcf3 function results in up-regulation of *siamois* and *Xnr3,* and the indirect targets *chordin* and *goosecoid,* both ectopically and in the organizer (Houston et al., 2002). One possibility is that one of the other XTcfs activates *siamois* and *Xnr3* expression, and acts more effectively when XTcf3 is depleted.

We addressed this question here by examining the function of maternal XTcf1 and XTcf4. We determined by explant and in situ hybridization analyses that XTcf1 represses Wnt target gene expression ventrally and laterally, and activates their expression dorsally. Double depletions of XTcf1 and XTcf3 suggest that they act cooperatively to repress Wnt target genes ventrally. In contrast, XTcf4 has no repressive role but is required to activate expression of *Xnr3* and *chordin* in organizer cells at the gastrula stage. This work provides evidence for distinct roles for XTcfs, and suggests that not all Tcf family members act as simple molecular switches.

Materials and methods

Xenopus laevis oocyte and embryo manipulation

Manually defolliculated oocytes were injected with oligonucleotides or mRNA, cultured at 18°C in oocyte culture medium (OCM), and fertilized using the host transfer technique as described (Zuck et al., 1998). Eggs were stripped and fertilized using a sperm suspension, and the embryos were maintained in 0.1× MMR. For injections after fertilization, embryos were dejellied (2% cysteine in 0.1× MMR, pH 8) and transferred into 2% Ficoll in 0.5× MMR prior to injection, then placed back into 0.1× MMR at stage 9. Embryos were staged according to (Nieuwkoop and Faber, 1967). For embryo bisections, the dorsal cells were marked at the 4-cell stage using Nile Blue (Sigma) precipitate in 2% Ficoll 0.1× MMR. At this stage, the dorsal and ventral cells can be clearly distinguished from one another on the basis of size and pigmentation. Embryos were bisected in 1× MMR at stage 10, when the dorsal lip had just become visible in the uninjected control embryos. The XTcf-depleted embryos were bisected at the same time as the controls on the basis of the Nile Blue marking, irrespective of whether the dorsal lip was visible. Dorsal and ventral halves were frozen for analysis at stage 10.25 and 2 h later at stage 11. Oocytes for bisection were fixed in ice cold 98% ethanol 2% acetic acid, then two cuts were made with a scalpel blade parallel to the equator and the narrow equatorial slice discarded. Animal and vegetal portions were washed several times in 100% ethanol and then RNA prepared as described (Kofron et al., 2001).

Oligonucleotides, mRNAs, and DNAs

The oligonucleotides used here were HPLC purified phosphodiester or phosphorothioate/phosphodiester chimeric oligos (phosphorothioate bonds are marked with an asterisk), of the following composition:

XTcf1: XTcf1.1, 5′-C*G*C*GACGGATCGCCT*C*G*G-3′
XTcf3: XTcf3.1, 5′-G*A*G*ATAACTCTGA*T*G*G-3′
XTcf4: XTcf4.1, 5′-C*A*G*GCTGTCCCGACT*C*T*T-3′
XTcf4.2, 5′-AACTTCCAGCCTGAACAT C-3′
XTcf4.3, 5′-TCTGCCTGTAAGTGCGGT-3′

Oligos were resuspended in sterile filtered water and injected into the vegetal poles of oocytes at doses stated in the text.

A clone (L1E6d05) containing the *Xenopus tropicalis* XTcf1C complete protein coding sequence (including a β-catenin binding domain) and 3′ UTR in the *Eco*RI/*Not*I site of pCS107 was a gift from Dr. A. Zorn. A *Hin*dIII/*Nae*1 fragment containing only the coding region from this plasmid was cloned into the *Stu*I site of pCS2+. *XTcf1* mRNA was synthesized in vitro from *Not*I-linearized template using the SP6 mMessage mMachine kit (Ambion). *XTcf3* mRNA was synthesized as described (Houston et al., 2002). pCS2 plasmids containing the *Xenopus laevis XTcf4A*, *4B*, and *4C* coding region sequences were a gift from Dr. D. Wedlich. *XTcf4* mRNA was synthesized *in vitro* from *Not*I-linearized template using the SP6 mMessage mMachine kit (Ambion). All RNAs were resuspended in sterile filtered water.

Analysis of gene expression using real-time RT-PCR

Total RNA was prepared from oocytes and embryos and cDNA synthesized as described (Kofron et al., 2001). Each sample frozen for mRNA extraction prior to RT-PCR analysis consisted of two whole embryos, four embryo halves, or two progesterone-matured oocytes. mRNA from approximately one-sixth embryo (or equivalent tissue) was used for cDNA synthesis with oligo (dT) primers. Real-time RT-PCR and quantitation using the LightCycler™ System (Roche) was carried out as described (Kofron et al., 2001). 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 served as a loading control. Samples of water alone or controls lacking reverse transcriptase in the cDNA synthesis failed to give specific products in all cases.

Primer sequences for all genes analyzed here are listed in (Tao et al., 2005), with the exception of *XTcf3* and *XTcf4*, given in (Houston et al., 2002), and the newly designed *XTcf1* primers, which were upstream, 5′-TACAGCCGTCA-CATGGTGTT-3′ and downstream, 5′-CAACTCCTCCAGAAGGCAAG-3′.

Wholemount in situ hybridization and lineage tracing

Chordin plasmid template consisting of the coding region inserted in the *Eco*RI/*Not*I site of pBluescript SK-was *Eco*RI linearized and digoxygenin-substituted antisense RNA probe was transcribed using T7 RNA polymerase. Embryos were fixed for 2 h in MEMFA and stored in methanol at −20°C. They were processed as described in (Harland, 1991) and visualized using BM Purple substrate (Roche). After sufficient color development they were re-fixed in MEMFA for 1 h, washed with methanol, bleached overnight in 10% hydrogen peroxide in methanol, then washed and stored in methanol at −20°C.

For lineage tracing, 500 pg *β-galactosidase* mRNA was injected into one B1 cell at the 32-cell stage. Embryos were fixed for 30 min in MEMFA and stained with red-gal (Research Organics, Inc.) as described in (Tanegashima et al., 2004). They were then re-fixed for 2 h in MEMFA and processed as above for *chordin* expression.

XTcf4 splice variant PCR

The primers used to amplify the region divergent between the three *XTcf4* splice variants were, upstream, 5′-GATCCCCCATCCGCTAGG-3′, and downstream, 5′-GCACCATGTGAGGAGGGAAT-3′. cDNA for this PCR reaction was primed using a gene specific primer just 3′ to the downstream primer, 5′-TTGTGTGCAAACTGTGATGA-3′. PCR products were electrophoresed on a 15% polyacrylamide gel (BioRad) and compared to a standard size marker to confirm their identity.

Luciferase assays

The *siamois* promoter-luciferase reporter DNA construct (pSia-Luc) was a gift from Dr. S. Sokol (Mount Sinai Medical Center, USA). It consists of 833 bp of the *siamois* promoter taken immediately upstream of the transcription start site and subcloned into a promoterless firefly (*Photinus pyralis*) luciferase reporter plasmid. This construct is described in (Fan et al., 1998). The plasmid pRLTK, consisting of *Renilla reniformis* luciferase and a ubiquitously active thymidine kinase (TK) promoter, also a gift of Dr. S. Sokol, was used as an internal control.

A total of 50 pg pSia-Luc together with 25 pg pRLTK DNA ± 1 ng *XTcf* mRNA was injected ventral-animally into two cells of embryos at the four cell stage. Three replicate samples of five embryos were frozen for each experimental group at stage 10.25–10.5, and luciferase assays performed using the Promega luciferase assay system. Each sample was homogenized in 100 μl lysis buffer and

cleared by microcentrifugation at 4°C. 50 μl substrate was added to 20 μl supernatant and luciferase activity measured for 10 s using a Luminescence Laboratory Monolight 2010 luminometer. Relative luciferase activity (*Photinus* luciferase activity normalized to *Renilla* luciferase activity) was determined for each sample. This experiment was carried out three times, and histograms show the mean of each three-reading set for a representative experiment.

Results

The expression of XTcf1 and XTcf4 in early Xenopus embryos

Xenopus embryos inherit maternal *XTcf1*, *XTcf3* and *XTcf4* mRNAs (Houston et al., 2002; Roel et al., 2003). Since XTcf4 exists in three isoforms (Pukrop et al., 2001), we designed PCR primers to amplify the divergent regions of the three variant forms from gene-specifically primed *Xenopus* oocyte cDNA, to determine which isoforms are expressed maternally. Fig. 1A shows that *XTcf4A* and *4B* predominate in oocytes, compared to tailbud stage embryos which express equal amounts of all three variants. Therefore, in our rescue experiments described below, we coinjected equal amounts of *XTcf4A* and *4B* mRNA. For our depletion experiments, we designed antisense oligos that were complementary to all isoforms of *XTcf4 (A–C)* or *XTcf1 (A–E)*, and thus would deplete all the *XTcf4* or *XTcf1* mRNAs present maternally.

Although the maternal expression of *XTcf1* and *XTcf4* has already been reported (Houston et al., 2002; Roel et al., 2003), and *XTcf1* has been shown by in situ hybridization to be expressed in the animal half of the early embryo (Roel et al., 2003), we wanted to confirm this asymmetry of expression, since in situ hybridization probes penetrate poorly into the yolky vegetal mass of *Xenopus* oocytes and early embryos. Therefore, we hemisected oocytes into animal and vegetal halves and compared expression of the three *XTcfs* by real-time RT-PCR analysis. Typically, mRNAs are more prevalent in the animal hemisphere of oocytes (Phillips, 1982), as seen here for *ODC* (Fig. 1B). *XTcf1* and *XTcf3* have a similar animal bias of expression; in comparison, *XTcf4* mRNA is equally distributed between the animal and vegetal hemispheres. None of the *XTcf* mRNAs are excluded from the vegetal hemisphere.

To determine if there is any dorsal/ventral bias of expression of the *XTcf* mRNAs in the early embryo, we analyzed batches of dorsal and ventral half embryos, frozen at the 32-cell stage. No significant differences were detected, suggesting that the three *XTcf* mRNAs are relatively ubiquitously expressed in the early embryo (data not shown).

Over-expression of XTcf1 or XTcf4 mRNA causes opposite effects on Wnt target gene expression

All three XTcfs contain β-catenin interaction motifs, and therefore might be expected to act similarly when over-expressed in the early embryo. To test this, a dose range of *XTcf1* or *XTcf4* mRNA was injected into oocytes to generate a uniform distribution of mRNA before fertilization. Control embryos, and embryos developing from *XTcf1*-overexpressing oocytes, were bisected into dorsal and ventral halves at the early gastrula stage for analysis of Wnt target gene expression, and the phenotypes of siblings were examined at the tailbud stage. Figs. 2A–C show that these embryos have a classically 'dorsalized' phenotype, as measured both by the up-regulation of Wnt target genes, and by their later appearance. In contrast, over-expression of *XTcf4* mRNA has the opposite effect, causing down-regulation of Wnt target genes and ventralized phenotypes (Figs. 2D–F). XTcf1 over-expression specifically causes the up-regulation of *Xnr3*, *goosecoid* and *chordin* in a dose responsive fashion both dorsally and ventrally, and does not affect the expression of the ventral marker *BMP4*. XTcf4 over-expression causes the down-regulation of *siamois*, *Xnr3*, *goosecoid*, and *chordin* and does not affect the expression of *BMP4*.

To confirm the different activities of XTcf1 and XTcf4, we next tested the ability of the *XTcf* mRNAs to activate a *siamois*-luciferase reporter, where luciferase is driven by 833 bp of the promoter of the endogenous *siamois* gene (Fan et al., 1998). In these experiments *XTcf1* or *XTcf4* mRNA was coinjected with the *siamois*-luciferase reporter construct into ventral animal regions at the 4-cell stage. Ventral animal cells were chosen as a site where early Wnt signaling does not normally occur and Wnt target genes are not expressed. In three experiments the

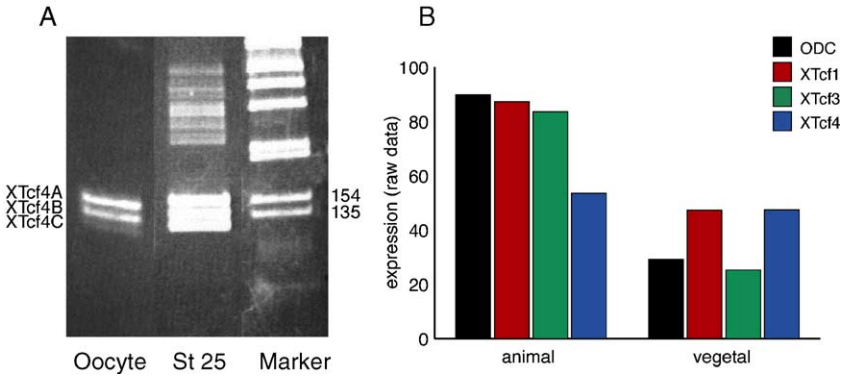

Fig. 1. (A) PCR was used to amplify the region of the cDNA that differs between the three splice variants of *XTcf4*, and the products were separated by gel electrophoresis. The expected fragment sizes were *XTcf4A*: 154, *4B*: 139, *4C*: 127 bp *XTcf4A* and *4B* predominate in oocytes (first lane), compared to tailbud stage embryos (second lane) which express equal amounts of all three variants. (B) Distribution of maternal *XTcf* mRNAs between animal and vegetal oocyte halves, shown by RT-PCR. *XTcf1* and *XTcf3*, like *ODC*, are predominantly localized to the animal hemisphere, while *XTcf4* is more equally distributed. Expression levels were not normalized but are relative to uninjected whole oocytes (not shown).

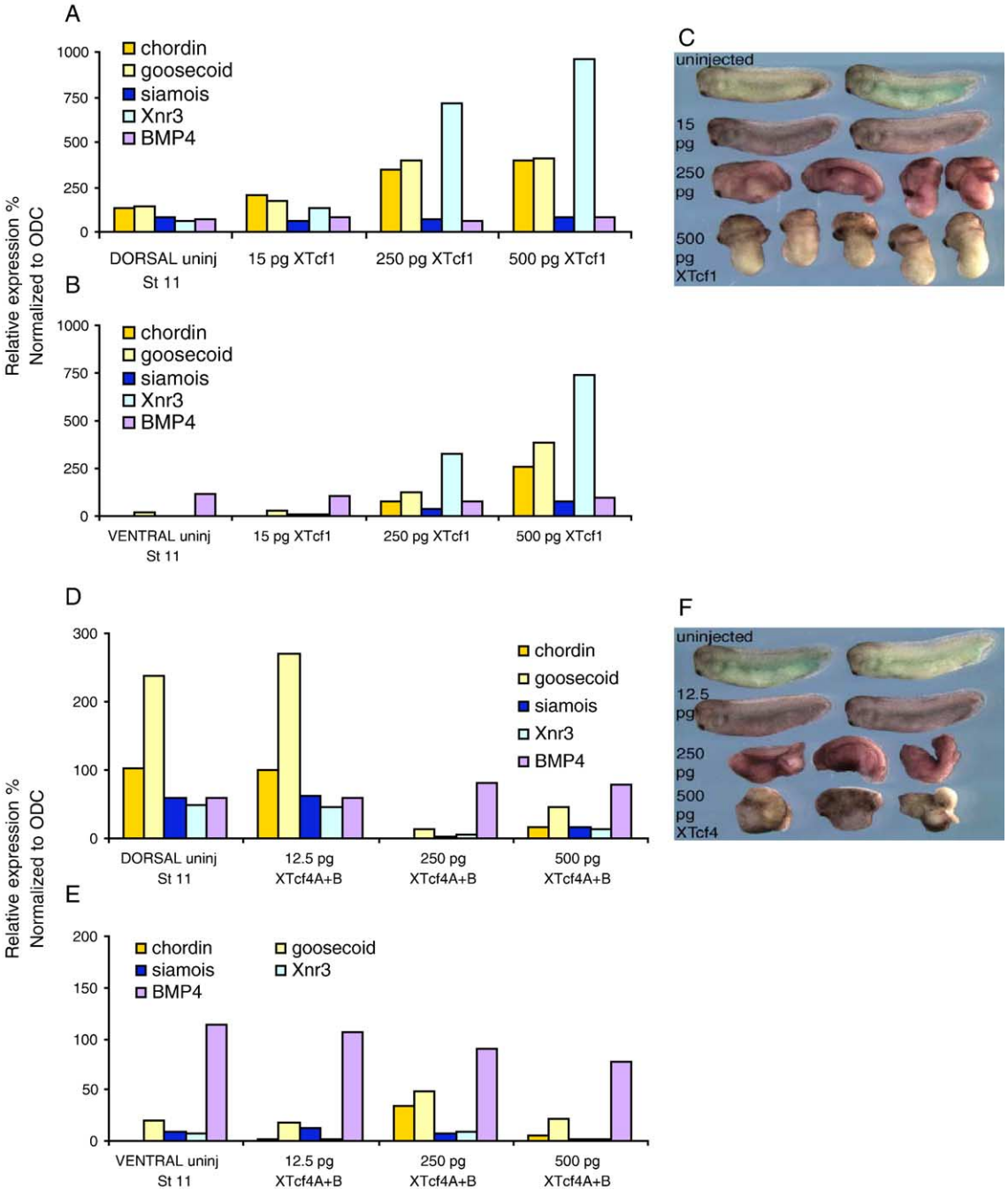

Fig. 2. Over-expression of XTcf1 and XTcf4 cause opposite effects on Wnt target gene expression. (A, B) Real time RT-PCR showing the relative expression of *chordin*, *goosecoid*, *siamois*, *Xnr3* and *BMP4* in (A) dorsal and (B) ventral half-embryos at the mid-gastrula stage. Embryos were uninjected controls or had been injected with 15 pg, 250 pg or 500 pg *XTcf1* mRNA as oocytes. Increasing doses of *XTcf1* mRNA cause increasing expression of Wnt target genes but not *BMP4*. (C) Sibling embryos to those in (A) and (B) at the tailbud stage. 250 pg and 500 pg *XTcf1* mRNA cause dorsalized phenotypes. (D, E) Real time RT-PCR showing the relative expression of *chordin*, *goosecoid*, *siamois*, *Xnr3* and *BMP4* mRNAs in (D) dorsal and (E) ventral half-embryos at the mid-gastrula stage. Embryos were uninjected controls, or had been injected with 12.5 pg, 250 pg or 500 pg *XTcf4A + B* mRNA as oocytes. Increasing doses of *XTcf4* mRNA cause reduced expression of Wnt target genes but not *BMP4*. (F) Sibling embryos to those in (D) and (E) at the tailbud stage. 250 pg and 500 pg *XTcf4* mRNA cause ventralized phenotypes.

expression of *XTcf1* mRNA in ventral cells caused a significant increase in the activity of the *siamois*-luciferase reporter compared to its activity alone, while *XTcf4A* and *4B* mRNAs did not (Fig. 3). Taken together, these studies suggest that *XTcf1* has the ability to activate Wnt target genes in the absence of a Wnt signal, and *XTcf4* does not.

Endogenous XTcf1 has dual roles in Wnt target gene expression

To determine the role of endogenous maternal XTcf1, we designed an antisense oligonucleotide that depletes *XTcf1* mRNA to less than 20% of control levels while not

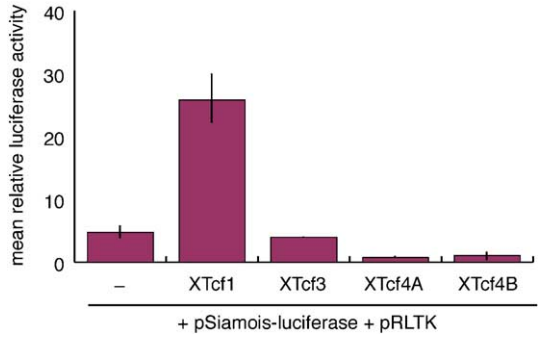

Fig. 3. *XTcf1*, but not *XTcf4*, stimulates a *siamois* promoter-luciferase reporter construct. Relative luciferase activity (pSia-Luc activity normalized to the pRLTK standard) was determined for 3 samples and each bar represents the mean of those results; the error bar extends one standard deviation either side of the top of each bar. Injections were directed to the animal pole of both ventral cells at the 4-cell stage. Analysis was carried out at stage 10.25. Injection amounts per embryo were 50 pg pSia-Luc, 25 pg RLTK, 1 ng *XTcf* mRNA.

affecting the expression of *XTcf3* or *XTcf4* (Fig. 4A). In a temporal expression series, zygotic *XTcf1* mRNA begins to be expressed at the early gastrula stage in embryos derived from maternal XTcf1 depleted oocytes. At the tailbud stage, *XTcf1* mRNA expression has recovered to control levels (Fig. 4B). In a temporal series of wild-type sibling controls and XTcf1-depleted embryos frozen at 2-h intervals from the late blastula stage we found that the expression of the direct Wnt target genes, *Xnr3* and *siamois* are initially delayed in expression compared to controls (data not shown).

Previously, we have shown that depletion of maternal XTcf3 causes up-regulation of Wnt target genes both in the organizer and ectopically in the ventral cells of the gastrula stage embryo (Houston et al., 2002). Therefore, rather than examining whole embryos here, we analyzed the effect of XTcf1 depletion on the expression of Wnt target genes in dorsal and ventral halves of XTcf1-depleted embryos, cut and immediately frozen at early and late gastrula stages. For XTcf1 depleted embryos, the dorsal/ventral axis was marked at the 8 cell stage using Nile blue crystals to ensure the correct plane of section at the gastrula stage. To determine whether the effects were specific, we injected back 15 pg *XTcf1* mRNA, since, in preliminary experiments, we found that doses above 60 pg *XTcf1* mRNA caused dorsalization when over-expressed. Figs. 5A and B show that XTcf1-depleted embryos have reduced expression of Wnt target genes dorsally and concomitant increased expression of Wnt target genes ventrally compared to controls. Similar effects were seen in four separate experiments. Furthermore, both changes were significantly rescued by the reintroduction of *XTcf1* mRNA, showing that the changes in gene expression are specifically due to XTcf1 depletion.

Sibling XTcf1-depleted embryos showed delayed gastrulation and a range of abnormalities at the tailbud stage (Fig. 5C), which were less severe than those seen in XTcf3-depleted embryos (Houston et al., 2002). Unfortunately, we do not have antibodies sensitive enough to detect endogenous protein, so we cannot distinguish whether this less penetrant phenotype reflects the fact that XTcf1 does not contribute as significantly as XTcf3 to embryonic patterning, or whether the depletion is incomplete and some stable maternal XTcf1 protein remains functional. These results suggest that maternal XTcf1 has dual roles in Wnt target gene expression, acting as a transcriptional repressor ventrally and activator dorsally.

XTcf1 and XTcf3 are interdependent transcriptional repressors

Since depletion of either *XTcf1* or *XTcf3* mRNA causes ectopic up-regulation of Wnt target genes in ventral cells, we asked whether they act cooperatively or independently. If each

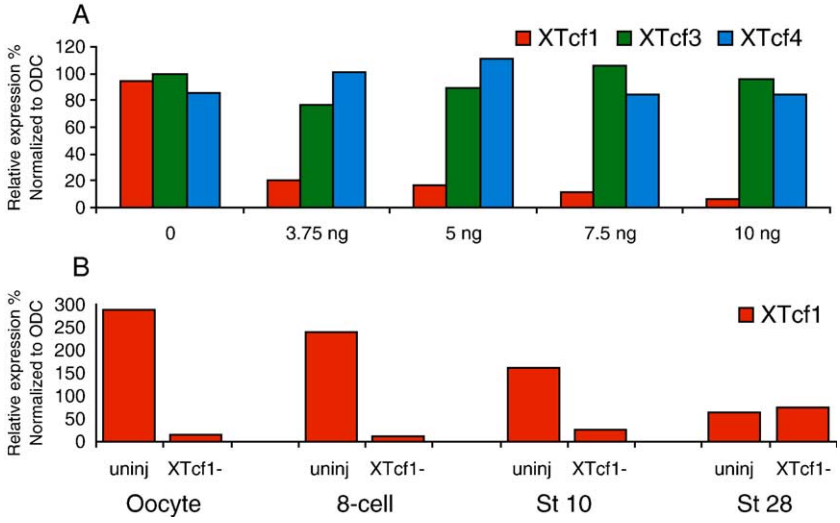

Fig. 4. (A) Real time RT-PCR shows that increasing doses of an antisense oligonucleotide directed against *XTcf1* mRNA depletes *XTcf1* mRNA to less than 20% of control levels while not affecting *XTcf3* or *XTcf4*. (B) In a temporal expression series of control and XTcf1-depleted oocytes and embryos, zygotic *XTcf1* begins to be expressed in maternally depleted oocytes at the early gastrula stage (stage 10). At the tailbud stage (stage 28), *XTcf1* mRNA expression in maternally depleted embryos is back to control levels.

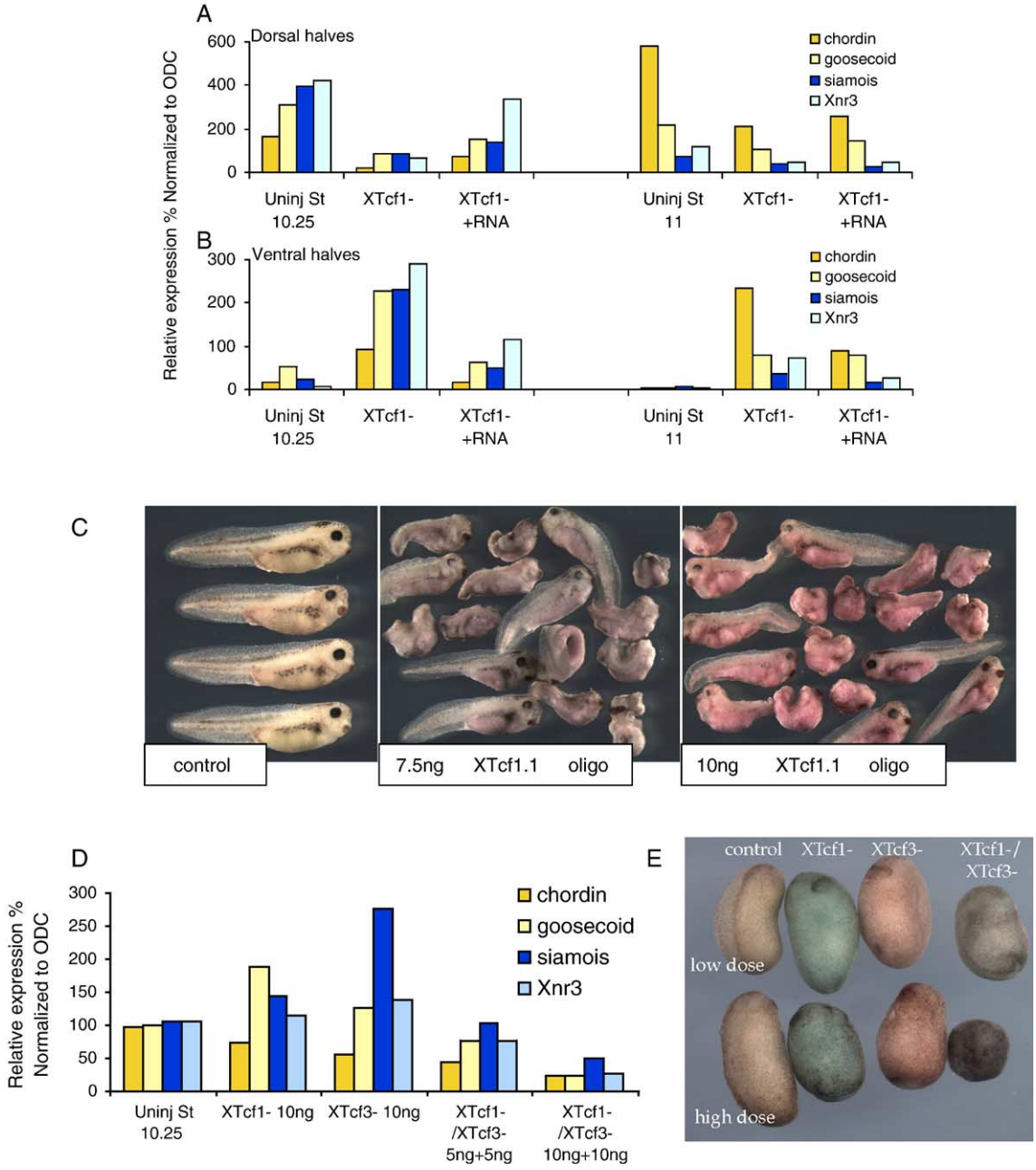

Fig. 5. The effects of maternal XTcf1 depletion. (A, B) Wnt target gene expression in bisected embryos at the early gastrula stage (stage 10.25) and 2 h later (stage 11). Real time RT-PCR showing the relative expression of *chordin*, *goosecoid*, *siamois*, and *Xnr3* in (A) dorsal and (B) ventral half-embryos. Embryos were uninjected control (uninj), injected with 7.5 ng XTcf1.1 oligo (XTcf1), or injected with 7.5 ng XTcf1.1 oligo and 48 h later with 15 pg *XTcf1* mRNA (XTcf1—+RNA). *XTcf1* depletion causes a loss of expression of Wnt target genes dorsally, and an increase in Wnt target gene expression ventrally. Both changes are partially rescued by the re-introduction of *XTcf1* mRNA. (C) The phenotypes of tadpoles injected as oocytes with 7.5 ng and 10 ng of XTcf1.1 (mauve and red, respectively) compared to uninjected control siblings (brown). (D) Real time RT-PCR to show the relative expression of Wnt target genes in early gastrula embryos derived from oocytes injected with 10 ng of XTcf1.1 oligo (XTcf1—10 ng); 10 ng Xtcf3.4 oligo (XTcf3—10 ng); 5 ng XTcf1.1 oligo + 5 ng XTcf3.4 oligo (XTcf1/XTcf3—5 ng + 5 ng); and 10 ng XTcf1.1 oligo + 10 ng XTcf3.4 oligo (XTcf1/XTcf3—10 ng + 10 ng). The depletion of *XTcf1* and *XTcf3* together causes a down-regulation of *chordin*, *goosecoid*, *siamois* and *Xnr3*. (E) The appearance of embryos derived from oocytes depleted of *XTcf1* alone, *XTcf3* alone or both together at low and high doses (7.5 ng + 7.5 ng).

acts independently of the other, either on the promoter of the same gene within individual cells, or in different cells, then depletion of both XTcf1 and XTcf3 should result in a further increase in the expression of Wnt target genes. This was not the case. Fig. 5D shows that XTcf1/3-double depleted embryos have much reduced expression of *siamois* and *Xnr3* compared to controls, suggesting that they act cooperatively and not independently. The experiment was repeated with a similar result. This indicates that the remaining activity of the other XTcf is required for the ectopic up-regulation of the Wnt targets outside the organizer, when either XTcf1 or XTcf3 is depleted.

XTcf4 does not act as a molecular switch for Wnt target gene function

To examine the role of XTcf4 on Wnt target gene expression, we depleted the maternally stored mRNA from oocytes using three different antisense oligonucleotides, all of which targeted both the maternal *XTcf4A* and *4B* isoforms and did not deplete *XTcf1* or *XTcf3* (Fig. 6A). Maternal *XTcf4* mRNA is much less abundant than *XTcf1* mRNA, and embryos are very sensitive to XTcf4 depletion, such that doses of each oligo that reduce the oocyte mRNA below 40% arrest the embryos at fertilization (data not shown). Therefore, we examined the effects of depleting oocyte mRNA levels to approximately 50% of control levels. *XTcf4* mRNA continued to decline during gastrulation, indicating that, unlike *XTcf1*, zygotic transcription of *XTcf4* does not commence until after the gastrula stage (Fig. 6B).

Fig. 7A shows that depletion of maternal XTcf4 causes a reduction in expression of the Wnt target genes *chordin* and *Xnr3*, although expression of *siamois* was merely delayed compared to controls. These results were highly reproducible in six experiments, and the reduced expression was partially rescued by the reintroduction of *XTcf4A+4B* mRNA into XTcf4-depleted oocytes. Zygotic genes known to be important in anterior specification, including *Xhex*, *cerberus* and *dickkopf* were also reduced in their expression levels (Fig. 7A and data not shown). At the tailbud stage, sibling embryos showed a loss of head structures (Fig. 7B; 14/21 cases ventralized) consistent with the loss of organizer gene expression, a phenotype that was partially rescued by the reintroduction of *XTcf4* mRNA (Fig. 7B; 6/20 cases ventralized). The experiment was repeated with a similar result. Embryos were extremely sensitive to the rescuing dose of mRNA. 12.5–25 pg of mRNA was most effective, while 50 pg or more exacerbated the ventralized phenotype (data not shown). Taken together, these results indicate that maternal XTcf4 is required for activation of Wnt target gene expression, as well as expression of anterior endodermal genes, and is essential for head formation. Unlike XTcf3, XTcf4 is not essential as a transcriptional repressor ventrally.

Finally, to confirm the different regulatory roles of XTcf1 and XTcf4, we examined the expression of *chordin* by in situ hybridization in sibling embryos derived from control, XTcf1- and XTcf4-depleted oocytes. Fig. 7C shows the expansion of *chordin* expression into the lateral and ventral marginal zone of XTcf1-depleted embryos, and the loss of *chordin* expression in the organizer of XTcf4-depleted embryos. Fig. 7D illustrates the diminished dorsal expression of *chordin* in XTcf1-depleted early gastrula compared to control, as well as its ectopic expression ventrally.

As *chordin* expression is reduced in dorsal halves of bisected XTcf1-depleted embryos as well as increased ventrally, we tested the possibility that XTcf1-depleted embryos had reversed dorsoventral polarity. We injected *β-galactosidase* mRNA into one B1 cell (dorsal animal, fated to contribute to the area above the dorsal lip at the early gastrula stage, (Bauer et al., 1994)) of 32-cell stage uninjected control and XTcf1-depleted embryos. The embryos were fixed at the mid-gastrula stage, stained with red-gal to color descendents of the *β-galactosidase* injected cells, and then processed to reveal *chordin* expression by situ hybridization. Fig. 7D shows that there was no reversal of dorsoventral polarity, indicating that the depletion of XTcf1 leads to the ectopic expression of *chordin* during gastrulation, but not a switch in dorsal versus ventral specification.

Discussion

In this work we aimed to analyze the roles of the maternal XTcfs, XTcf1 and XTcf4, in early *Xenopus* development. Several previous loss of function studies have shown the importance of Tcfs in vertebrate development. In particular

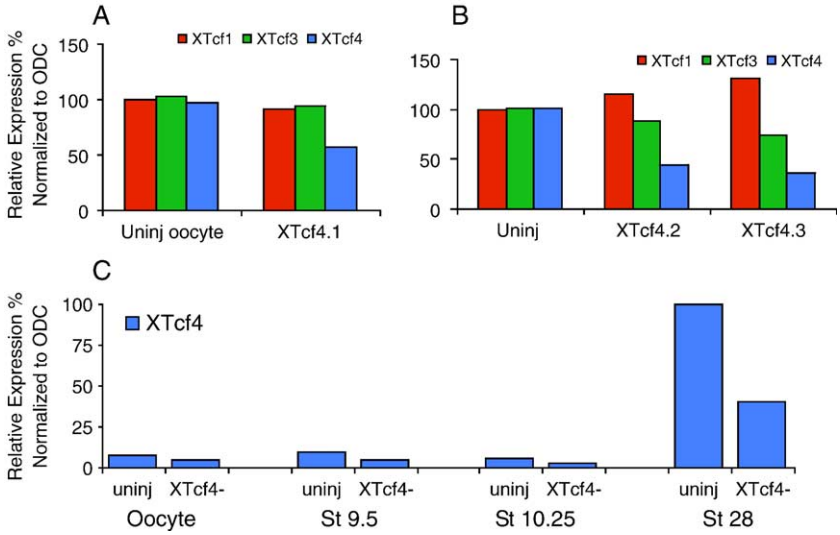

Fig. 6. (A, B) Real time RT-PCR shows that the three antisense oligonucleotides directed against *XTcf4* mRNA deplete all splice variants of *XTcf4* mRNA to 40–50% of control levels while not affecting the expression of *XTcf1* or *XTcf3*. (C) In a temporal expression series of control and XTcf4-depleted oocytes and embryos, *XTcf4* mRNA levels in embryos derived from oocytes injected with 7 ng Tcf4.1 oligo remain lower than control embryos at tailbud stage (stage 28).

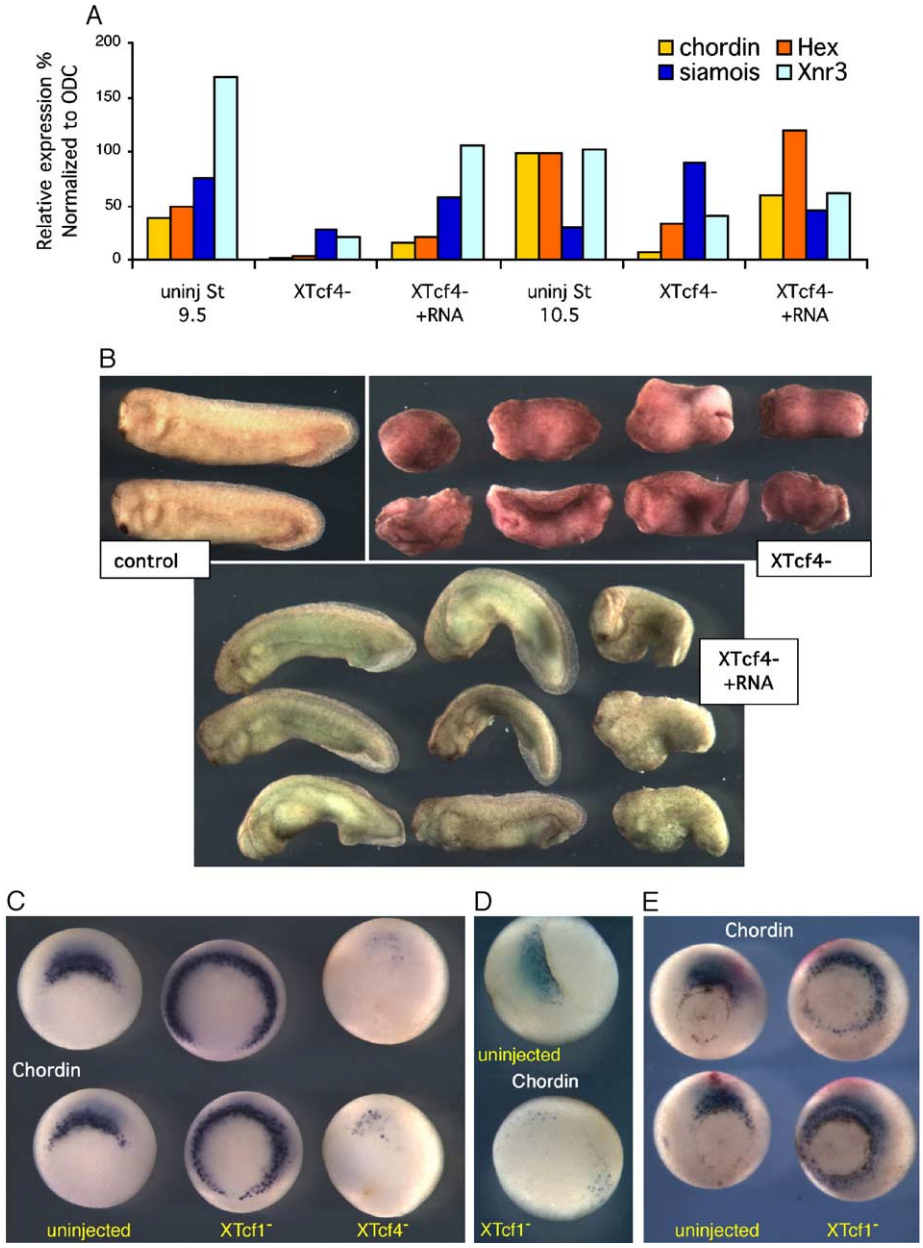

Fig. 7. The effects of maternal XTcf4 depletion. (A) Wnt target gene expression in whole embryos at the late blastula (stage 9.5) and early gastrula stage (stage 10.5). Real time RT-PCR showing the relative expression of *chordin*, *Xhex*, *siamois*, and *Xnr3*. Embryos were uninjected controls (uninj), injected with 4 ng *XTcf4.1* oligo (XTcf4−), or injected with 4 ng XTcf4.1 oligo and 48 h later with 12.5 pg *XTcf4A* mRNA (XTcf4−+RNA). *XTcf4* depletion causes a loss of expression of Wnt target genes, particularly *chordin*, *Xhex*, and *Xnr3*, changes that are partially rescued by the re-introduction of *XTcf4* mRNA. (B) The phenotypes of tailbud stage embryos injected as oocytes with 7 ng XTcf4.1 oligo (red) compared to uninjected control siblings (brown). The ventralized phenotype is partially rescued by the introduction of 12.5 pg *XTcf4A* + *4B* mRNA (blue). (C) *Chordin* expression extends further ventrally in XTcf1-depleted embryos (center) than in uninjected controls (left). *Chordin* expression is greatly diminished in XTcf4-depleted embryos (right). Vegetal view of stage 10.5 embryos, dorsal at top. (D) *Chordin* expression is much diminished in XTcf1-depleted early gastrulae compared to uninjected controls at the early gastrula stage. (E) Vegetal view of stage 10.5 control embryos (left) and XTcf1^{-} embryos (right), dorsal at top. *β-galactosidase* mRNA injected into cell B1 at the 32-cell stage marks dorsal tissue in the gastrula, visualized in red. The red stain colocalizes with the most intense expression of *chordin*, visualized in blue, in all the embryos, showing that there has been no reversal of dorsoventral polarity as a result of XTcf1 depletion.

mouse, *Xenopus* and zebrafish studies have defined the repressive function of Tcf3 (Dorsky et al., 2003; Houston et al., 2002; Merrill et al., 2004). Double knockouts of Tcf1/LEF1 and Tcf4/Tcf1 in mouse, showed the particular importance of the Wnt signaling pathway in patterning the caudal embryonic axis, the former showing particular deficiencies in the specification of paraxial mesoderm (Galceran et al., 1999), and the latter of the caudal endoderm (Gregorieff et al., 2004). These studies suggest the cooperative function of different Tcf family members in transducing Wnt signals. The relative roles of Tcf1, Tcf3 and Tcf4 in regulating the expression of individual target genes is not well understood. Here we addressed the question whether the loss of function of maternal XTcf1 and XTcf4 cause the same or different effects on the

expression of the direct target genes, *siamois* and *Xnr3*, and the indirect targets *chordin* and *goosecoid*.

One complexity of studying XTcf1 and XTcf4 function is that several isoforms exist in the early embryo. To simplify the question, we studied only the maternally expressed forms, by using antisense oligonucleotides injected into oocytes before fertilization. In these experiments, the antisense oligo and target mRNA are degraded before fertilization (over a 48-h culture period). Zygotic *XTcf1* and *XTcf4* are therefore not affected in these experiments. Since it had previously been determined that both *XTcf1* isoforms *C* and *E*, with distinct C-termini, are present maternally (Roel et al., 2003), and that both have β-catenin binding domains, we designed antisense oligos that depleted both forms. Similarly, we determined here that *XTcf4A* and *4B* isoforms predominate maternally, and designed oligos to deplete both (these oligos would also hybridize to any *XTcf4C* present).

The starting point for these experiments was that maternal β-catenin is absolutely required for the expression of Wnt target genes, that all the maternal XTcfs have β-catenin binding motifs, and that XTcf3 depletion causes an up-regulation of Wnt target gene expression in dorsal and ventral cells. Here we have added the findings that XTcf1 and XTcf4 depletion have different effects; XTcf1 is, like XTcf3, a repressor of ectopic Wnt target gene expression, but XTcf4 acts as an activator.

Over-expression studies are not reliable indicators of the roles of XTcfs in early embryos

Over-expression of *XTcf1* and *XTcf4* mRNAs in the oocyte caused extremely consistent effects on gene expression and patterning of the embryo; *XTcf1* caused up-regulation of the direct target *Xnr3*, but not *siamois,* while *XTcf4* over-expression caused a severe reduction in Wnt target gene expression. This finding suggests that XTcf1 acts as a transcriptional activator of Xnr3, overriding endogenous regulators, and does not regulate *siamois*, results that are not borne out by loss of function studies. The repression caused by XTcf4 over-expression could be a direct effect, or it could be acting as a dominant negative, sequestering β-catenin without DNA binding. This highlights one problem general to over-expression studies, that they may not elucidate the activity of the endogenous gene being studied. Tcfs clearly act by interaction with multiple binding partners, whose concentrations may be limiting, and whose activities may be distorted by an excess of exogenous Tcf. One indication of the sensitivity of the embryo to the level of Tcf expression, shown here, is that while 12.5 pg of *XTcf4* mRNA was sufficient to rescue embryos from the ventralized phenotype caused by XTcf4 depletion, 50 pg of *XTcf4* mRNA increased ventralization.

XTcf1 is required as a repressor of ectopic Wnt target gene expression, and as an activator of the same genes in the organizer

In previous experiments, we showed that XTcf3 depletion caused up-regulation of Wnt target genes both ectopically and in the organizer region. Here we show that XTcf1 depletion results in the same effect ventrally, an ectopic up-regulation of *siamois*, *Xnr3*, *chordin* and *goosecoid*, and the opposite effect, their down-regulation, dorsally. These effects are rescuable by the reintroduction of *XTcf1* mRNA before fertilization, indicating that they are specific to the loss of XTcf1. The *C. elegans* Tcf protein POP-1 has also recently been shown to function as both a repressor and activator of transcription (Shetty et al., 2005). While activation of Wnt target genes in the endoderm lineage was previously thought to be achieved simply by lowering nuclear levels of POP-1, thereby relieving its repressive function, the experiments of Shetty and colleagues demonstrate that Wnt signalling effectively converts POP-1 from a repressor to an activator. By lowering the nuclear concentration of POP-1, Wnt signalling changes the POP-1:coactivator ratio such that Wnt-responsive genes are transcribed. This induction requires the N-terminal β-catenin binding domain of POP-1, demonstrating that POP-1 has an activating role in transcription, not merely a repressive one.

The fact that the double depletion of XTcf1 and XTcf3 leads to a loss of Wnt target gene expression, rather than an increase, indicates that the up-regulation of *siamois* and *Xnr3* seen in single depletions is dependent on the other XTcf. For example, when XTcf1 is removed from ventral cells, the consequent up-regulation of *chordin* expression requires XTcf3. We know that the up-regulation of Wnt target gene expression is β-catenin dependent, since double depletions of β-catenin/XTcf3 are ventralized (data not shown). One possible model to explain this is that the removal of one XTcf also removes some groucho protein from its repressive location, thus swinging the β-catenin/groucho competitive antagonism in favor of β-catenin in ventral cells, even though there is no Wnt signaling enhancing nuclear accumulation of β-catenin ventrally. Although previous immunostaining experiments emphasized the dorsal asymmetrical accumulation of β-catenin in early cleavage stages, it is clear that β-catenin is also present ventrally in cleavage stage embryos ((Larabell et al., 1997; Schohl and Fagotto, 2002) and data not shown). Similarly, when XTcf3 is depleted dorsally, the presence of XTcf1, and the reduced amount of groucho, may increase the chromatin remodeling activity of β-catenin and further activate the transcription of Wnt target genes.

One question that arises is why XTcf1 and XTcf3 depletions have different effects dorsally, the former resulting in a loss of target gene expression, and the latter an increase. In over-expression experiments, XTcf1 and XTcf3 also perform differently, the former activating Wnt target genes and the latter repressing them. This suggests that they may have different DNA binding affinities, while both bind β-catenin. In this model, XTcf1 has a higher DNA binding affinity than XTcf3. Alternatively, XTcf1 may bind more effectively to a co-activating partner than XTcf3. In this regard, XTcf1 resembles XTcf4, since when either is depleted the expression of Wnt target genes in the organizer is reduced. Interestingly, both XTcf1 and XTcf4 share a conserved CRARF motif in their C-termini, whose function

is currently not known, but is not present in XTcf3 (Hurlstone and Clevers, 2002).

XTcf4 does not act as a molecular switch

In the molecular switch model, Tcfs have repressive function because they bind HDACs such as groucho and CtBP in the absence of β-catenin (Daniels and Weis, 2005; Hurlstone and Clevers, 2002). β-catenin competitively antagonizes groucho binding, and converts the repressive activity into transcriptional activation, by recruiting chromatin remodeling proteins, and, in the case of XTcf3, allowing other transcriptional activators such as VegT and FoxH1 to function (Houston et al., 2002; Kofron et al., 2004). The loss of function experiments described here show that XTcf4 does not have any repressive activity, or, alternatively, that it is redundant with the other XTcfs in this role. One caveat of this experiment was that *XTcf4* mRNA levels were reduced by only 50%. However, the fact that XTcf3 depletion causes up-regulation of Wnt target genes in the organizer, even though XTcf4 is still present, argues against XTcf4 having a repressive function redundant with XTcf3. The fact that XTcf4 acts as an activator of *Xnr3* is unexpected, since it has groucho and CtBP binding domains, and has been shown in cell culture experiments to repress expression of the *fibronectin* gene (Gradl et al., 1999). It is likely that XTcf4 activity is extremely promoter specific, and determined by the position of its binding sequences relative to the basal transcription machinery, their precise sequence and the presence of other transcription factors. In studies of the mouse and human *matrix metalloproteinase* (*MMP*) promoters, both the binding sequence and the position of the binding sites determined the type of responsiveness, either activation or repression, to Tcf/LEF binding (Gustavson et al., 2004). Taken together, these results suggest that all three maternally expressed XTcfs play roles in regulating the zygotic expression of the Wnt target genes; XTcf1 and XTcf3 act cooperatively as ventral repressors, and XTcf1 and XTcf4 as dorsal activators.

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