Development 126, 3159-3170 (1999)
Printed in Great Britain © The Company of Biologists Limited 1999
DEV5316

XCtBP is a XTcf-3 co-repressor with roles throughout *Xenopus* development

Mark Brannon[1,*], Jeffrey D. Brown[2,*], Rebecca Bates[2], David Kimelman[1,‡] and Randall T. Moon[2,‡]

[1]Department of Biochemistry and [2]Howard Hughes Medical Institute and Department of Pharmacology, and [1,2]Center for Developmental Biology, University of Washington School of Medicine, Seattle, Washington 98195-7350, USA

*These authors contributed equally to the work

‡Authors for correspondence (e-mail: kimelman@u.washington.edu; rtmoon@u.washington.edu)

Accepted 3May; published on WWW 21 June 1999

SUMMARY

XTcf-3 is an HMG box transcription factor that mediates *Xenopus* dorsal-ventral axis formation. As a Wnt pathway effector, XTcf-3 interacts with β-catenin and activates the expression of the dorsal organizing gene *siamois*, while in the absence of β-catenin, XTcf-3 functions as a transcriptional repressor. We show that XTcf-3 contains amino- and carboxy-terminal repressor domains and have identified a *Xenopus* member of the C-terminal Binding Protein family of transcriptional co-repressors (XCtBP) as the C-terminal co-repressor. We show that two XCtBP binding sites near the XTcf-3 carboxy-terminus are required for the interaction of XTcf-3 and XCtBP and for the transcriptional repression mediated by the XTcf-3 carboxy-terminal domain. By fusing the GAL4 activation domain to XCtBP we have generated an antimorphic protein, XCtBP/G4A, that activates *siamois* transcription through an interaction with endogenous XTcf-3. Ectopic expression of XCtBP/G4A demonstrates that XCtBP functions in the regulation of head and notochord development. Our data support a role for XCtBP as a co-repressor throughout *Xenopus* development and indicate that XCtBP/G4A will be a useful tool in determining how XCtBP functions in various developmental processes.

Key words: CtBP, XTcf-3, Lef/Tcf, Transcriptional repression, Wnt pathway, *Xenopus*

INTRODUCTION

Studies in many systems have established the importance of the *Wnt* pathway in regulating numerous processes during embryonic development, as well as cell proliferation during later life (Cadigan and Nusse, 1997; Moon et al., 1997; Cox and Peifer, 1998; Wodarz and Nusse, 1998). A key step in the activation of Wnt-responsive genes is the binding of the transcriptional co-activator β-catenin/Armadillo to DNA-binding transcription factors of the Tcf/Lef family (Behrens et al., 1996; Huber et al., 1996; Molenaar et al., 1996; Brunner et al., 1997; van de Wetering et al., 1997). In early *Xenopus* development, for example, the *Wnt* pathway activates the expression of two homeobox genes, *siamois* (Lemaire et al., 1995; Brannon and Kimelman, 1996; Yang-Snyder et al., 1996) and *twin* (Laurent et al., 1997) and the signaling factor *Xnr3* (Smith et al., 1995) as an early step in the process that establishes the dorsal-ventral and anterior-posterior axes (Moon and Kimelman, 1998).

Siamois, *twin* and *Xnr3* contain Tcf/Lef binding sites in their promoters (Brannon et al., 1997; Laurent et al., 1997; McKendry et al., 1997; Fan et al., 1998), suggesting that the early dorsal accumulation of β-catenin in the *Xenopus* embryo leads to the direct activation of these genes (Larabell et al., 1996; Moon and Kimelman, 1998). Surprisingly, the elimination of all three Tcf/Lef binding sites from the *siamois* promoter results in the elevated expression of the gene (Brannon et al., 1997), indicating that Tcf/Lef proteins could function as repressors (reviewed by Bienz, 1998). Thus, the endogenous maternal *Xenopus* Tcf/Lef family member, XTcf-3 (Molenaar et al., 1996), was proposed to repress *siamois* on the ventral side of the embryo, whereas the enhanced dorsal levels of β-catenin were proposed to overcome this repression, leading to the dorsal expression of *siamois* (Brannon et al., 1997). Similar results were obtained in *Drosophila*, where the elimination of dTcf binding sites in the *Ultrabithorax* gene expanded its domain of expression (Riese et al., 1997). Moreover, mutants in the *Caenorhabditis elegans* Tcf homologue, *pop-1*, produce a phenotype equivalent to constitutive *Wnt* signaling, indicating that POP-1 also functions as a transcriptional repressor (Rocheleau et al., 1997; Thorpe et al., 1997).

In *Xenopus*, ectopic expression of XTcf-3 and mouse LEF-1 produce significantly different results. When XTcf-3 is ectopically expressed on the ventral side of *Xenopus* embryos, no alteration in development is observed (Molenaar et al., 1996). In contrast, ventral ectopic expression of LEF-1 results in the formation of a partial secondary axis (Behrens et al., 1996). Since β-catenin levels are low on the ventral side of *Xenopus* embryos (Larabell et al., 1996; Schneider et al., 1996), we suspected that the ectopically supplied LEF-1 might function by displacing the endogenous XTcf-3, an event which would derepress the *siamois* promoter if LEF-1 lacked the repressing function of XTcf-3.

We have used the functional difference between XTcf-3 and LEF-1 to identify two regions in XTcf-3, including the C-terminal domain, that possess transcriptional repressor activity. Using a yeast two-hybrid screen, we have identified XCtBP as a protein that binds two sites near the C terminus of XTcf-3 and is responsible for the repressing function of this domain. We then constructed an antimorphic form of XCtBP and used it to demonstrate the in vivo interaction between XCtBP and XTcf-3 in the regulation of *siamois*. Antimorphic XCtBP also reveals additional roles for XCtBP-mediated repression at later stages of development.

MATERIALS AND METHODS

Cloning of XCtBP

An amplified *Xenopus laevis* oocyte library in the vector pGAD10 (Clontech) was screened with full-length XTcf-3 cloned into pGBT9 according to the manufacturer's instructions (Clontech). The original complexity of the library before amplification was 2.5×10^6 independent clones. Approximately 2×10^6 colonies were screened. A single, full-length clone of XCtBP was identified among the putative interacting clones.

DNA constructs

The XCtBP clone was excised from pGAD10 by restriction with *Sal*I, cloned into the *Xho*I site of CS2P+ (CS2P+ was a gift from R. Davis) to produce XCtBP-CS2P+. To fuse the GAL4 activation domain in frame to the C terminus of XCtBP, XCtBP was amplified by PCR using the CS2+ SP6 primer and oligonucleotide MB27 (5′-CGCAGATCTCTGATCAGTAGGAATTTCTCTG-3′), which introduces a *Bgl*II site (underlined) and eliminates the XCtBP termination codon. The amplification product was directionally cloned into the *Bam*HI and *Bgl*II sites of CS2+G4A (kindly provided by D. Turner) to produce XCtBP/G4A-CS2+.

For in vitro synthesis of RNA, hLEF-1 and XTcf-3 were subcloned into CS2+ (Turner and Weintraub, 1994). hLEF-1 in pGdSS9BΔBE (kindly provided by M. Waterman) was digested with *Hin*dIII, blunted by filling with T4 DNA polymerase, digested with *Eco*RI, and directionally cloned into *Eco*RI and *Stu*I cut CS2+ to produce hLEF-1-CS2+. XTcf-3 in pT7Ts (a gift from O. Destrée) was digested with *Cla*I and *Spe*I and directionally cloned into *Cla*I and *Xba*I digested CS2+ to produce XTcf-3-CS2+.

To generate C-terminally truncated XTcf-3 (ΔC-XTcf-3), oligonucleotide MB2 (5′-GCGGAATT**C**T**A**CTTTTCTCTCTTCCG-3′), which introduces an *Eco*RI site (underlined) and a termination codon (in bold), was designed. Using MB2 and the SP6 primer, XTcf-3 was amplified by PCR. The amplification product was directionally cloned into the *Cla*I and *Eco*RI sites of CS2+ to produce ΔC-XTcf-3-CS2+.

Chimeras 1, 2 and 3 (fusions of XTcf-3 and hLEF-1) were generated by overlapping PCR. Oligonucleotides MB4 (5′-GCACCACGGGGCACTTTATTTG-3′), MB6 (5′-CAATAGCTGGATGAGGGATGC-3′) and MB11 (5′-CTCTCTCTTCCTCTTCTTTTTC-3′), which anneal to successively more 3′-regions of the hLEF-1 sequence, were used with the SP6 primer to amplify progressively larger regions of hLEF-1 by PCR. Oligonucleotides MB5 (5′-CAAATAAAGTGCCCGTGGTGCAGCACCCACACCACATGCACC-3′), MB7 (5′-GCATCCCTCATCCAGCTATTGTTTCGCCCATTGTGAAACAGGAG-3′) and MB12 (5′-GAAAAAGAAGAGGAAGAGAGAGAAGCAGTCACCTGAGATGG-3′), which contain overhangs (underlined) that anneal to oligonucleotides MB4, MB6 and MB11, respectively, were used with MB8 (5′-GCTCTAGAGATCGATCCCCGGTTGTCC-3'), which anneals to the 3′-end of XTcf-3 and introduces a *Xba*I site (underlined), to amplify progressively shorter regions of XTcf-3 by PCR. All amplification products were gel purified, combined (MB4 LEF-1 with MB5 XTcf-3, MB6 LEF-1 with MB7 XTcf-3 and MB11 LEF-1 with MB12 XTcf-3), reamplified with the flanking SP6 and MB8 oligonucleotides to produce full-length LEF-1/XTcf-3 fusions and directionally cloned into *Eco*RI and *Xba*I digested CS2+ to produce Chimeras 1, 2 and 3.

Chimera 3 mutants that lack XCtBP binding sites were produced as follows. Chimera 3 C-terminally truncated at XCtBP site 1 (Chimera 3/Δ-CtBP) was generated using oligonucleotide MB21 (5′-GCGTCTAGAA**TCA**CTGAGCTTGCTCAGAGTGAG-3′), which introduces a *Xba*I site (underlined) and a termination codon (in bold), with the SP6 primer to amplify Chimera 3 by PCR. The amplification product was directionally cloned into *Eco*RI and *Xba*I digested CS2+ to give Chimera 3/Δ-CtBP-CS2+. To produce Chimera 3 mutant in XCtBP site 2, oligonucleotide MB24 (5′-GCGTCTAGACTCAGTCACTGGATTTGGTCACCAGAGAA**GA**GG**C**CTGTGCCTGCAGCAG-3′), which introduces *Xba*I and *Stu*I sites (underlined) and base changes (in bold), was designed. By use of MB24 and the SP6 primer, Chimera 3 mutant in site 2 was amplified by PCR. The amplification product was cloned into *Eco*RI and *Xba*I digested CS2+ to produce Chimera 3/mutant 2-CS2+. Oligonucleotide-mediated, site-directed mutagenesis (Kunkel et al., 1987) was used to produce the Chimera 3 XCtBP site 1 and double mutants. The mismatched oligonucleotide MB25 (5′-CTCTGAGCAAGCT**C**A**G**G**C**CTCCTCCCTCACCACCA-3′), which introduces a *Stu*I site (underlined) and base changes (in bold) was used with Chimera 3 or Chimera 3/mutant 2 to generate Chimera 3/mutant 1-CS2+ and Chimera 3/double mutant-CS2+, respectively. All PCR was performed with *Pfu* polymerase (Stratagene) and all constructs were verified by sequencing.

Embryo manipulation, RNA synthesis and microinjection

Xenopus embryos were obtained by artificial fertilization of eggs as previously described (Pierce and Kimelman, 1995). Embryos were cultured at 14°C to 23°C and staged according to Nieuwkoop and Faber (1967). Dorsal and ventral marginal tissue and animal and vegetal tissue explants used for the RT-PCR assays were obtained by dissection of stage 10 embryos.

All constructs described above were linearized by digestion with *Not*I and synthetic RNAs were produced using the SP6 mMessage mMachine kit (Ambion).

Embryos were microinjected at the 4-cell stage into the equator of either dorsal or ventral blastomeres. The volume of each microinjection was 10 nl per blastomere. All RNAs were used at a final amount of 0.5 ng per embryo, except for the *XCtBP* and *XCtBP/G4A* RNAs which were used at a final amount of 1 ng and 0.2 ng, respectively. The *siamois* luciferase reporter plasmids, S01234 and S24, were used at a final amount of 225 pg per embryo.

Luciferase, RT-PCR and GST pull-down assays

Luciferase assays were performed as previously described (Brannon et al., 1997).

RT-PCR assays of *siamois* activation and analysis of *XCtBP* expression were performed as previously described (Cui et al., 1995; Brannon and Kimelman, 1996). PCR primers were as decribed; *EF-1α* (Hemmati-Brivanlou et al., 1994), *histone H4* (Niehrs et al., 1994), *Xwnt-8* and *Vg1* (Cui et al., 1995), *Xnr-3* and *siamois* (Yang-Snyder et al., 1996). Primers to the 5′-region of *XCtBP* were, forward (5′-CAGAGAAGTTGCTGGAGGGG-3′) and reverse (5′-CAGTAGCTCCTGAAGAGTCGCC-3′) and primers to the 3′-region of *XCtBP* were, forward (5′-GCTGCCGTTCATCCAGAAC-3′) and reverse (5′-TCAGGTTTGGTGGTTTGTCC-3′).

Pull-down assays using GST-dCtBP and ^{35}S-labeled normal and mutant forms of Chimera 3 were performed as described (Poortinga et al., 1998).

In situ hybridization and histology

Whole-mount in situ hybridization was performed using digoxigenin-labeled antisense RNA probes (Harland, 1991). The *Xnot* probe was

used as described previously (Schmidt et al., 1996). XCtBP-CS2P+ was linearized with *Bam*HI and transcribed with T7 RNA polymerase to produce the antisense probe and *Xba*I and SP6 for the sense probe.

For histological analysis, stage 20 and 33 embryos were formaldehyde fixed and dehydrated in absolute ethanol. After incubation in toluene, embryos were infiltrated with Paraplast X-tra (Oxford Labware) for 2 hours at 60°C. 8-12 μm sections were cut and adhered to Superfrost/Plus slides (Fisher Scientific) on a slide warmer at approx. 40°C overnight. Wax was removed and tissue subjected to hematoxylin and eosin staining (Kelly et al., 1991).

RESULTS

XTcf-3 is a transcriptional repressor and LEF-1 is not

The prospect that XTcf-3 functions as a transcriptional repressor (Brannon et al., 1997; Riese et al., 1997), combined with the dissimilar activities of ectopic XTcf-3 and LEF-1 in *Xenopus* embryos (Behrens et al., 1996; Huber et al., 1996; Molenaar et al., 1996), prompted us to examine the transcriptional regulatory activities of these two factors in more detail. We compared the activities of XTcf-3 and LEF-1 in *Xenopus* embryos using three assays. In each assay, synthetic RNA encoding XTcf-3 or LEF-1 was injected into the dorsal equatorial region of 4-cell stage *Xenopus* embryos. In the first assay, injected embryos were assigned a Dorso-Anterior Index (DAI) score (Kao and Elinson, 1988) at the tadpole stage, based on the extent of dorsoanterior structure formation and compared to uninjected control embryos (DAI=5.0; normal control). We expected that if XTcf-3 or LEF-1 behaves as a transcriptional repressor dorsal injection should result in embryos with low DAI scores (DAI=0; completely ventralized). In the second assay we injected the *siamois* promoter fused to a luciferase reporter gene (construct S01234; Brannon et al., 1997) into the dorsal equatorial region of early *Xenopus* embryos in the presence or absence of *XTcf-3* or *LEF-1* RNA. The *siamois* reporter gene is activated to high levels on the dorsal side of *Xenopus* embryos (Brannon et al., 1997), and we asked whether overexpression of *XTcf-3* or *LEF-1* RNA would affect this activation. The third assay utilized RT-PCR to measure the relative levels of endogenous *siamois* transcripts present in dorsal explants of control embryos or embryos injected with *XTcf-3* or *LEF-1* RNA.

The results of all three assays are consistent with XTcf-3 behaving as a transcriptional repressor, while LEF-1 appeared to have no repressor activity in early *Xenopus* embryos (summarized in Fig. 1A). Dorsal injection of 0.5 ng of *XTcf-3*

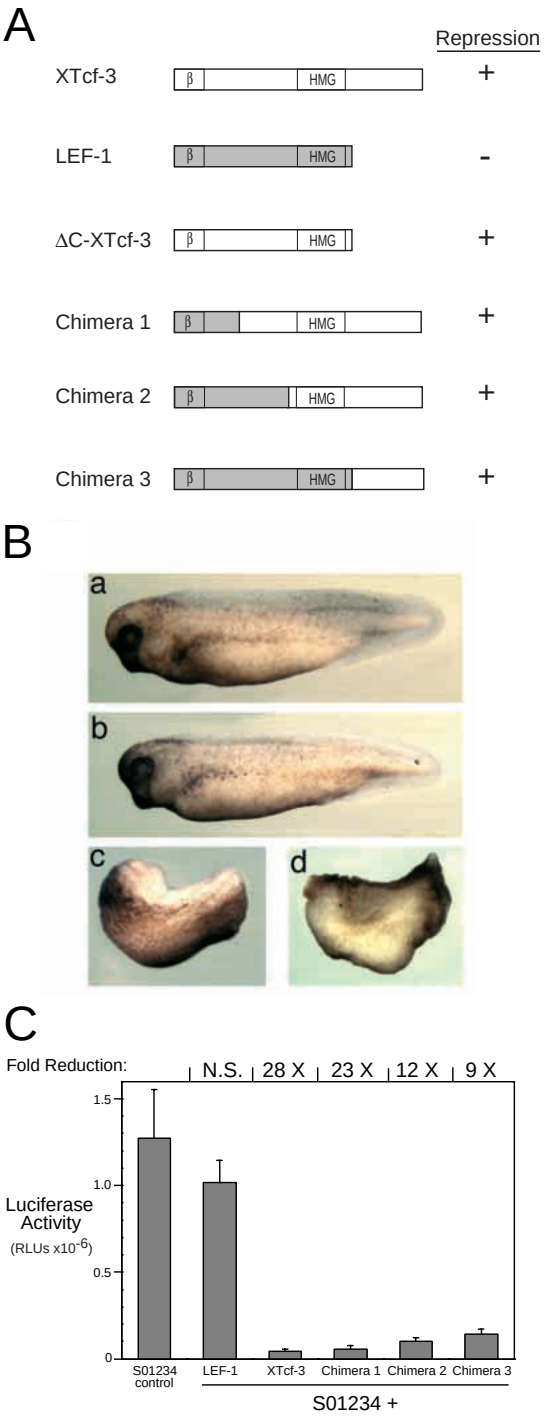

Fig. 1. XTcf-3 contains N- and C-terminal repressor domains. (A) Schematic diagram of XTcf-3, LEF-1, ΔC–XTcf-3 and the LEF-1/XTcf-3 chimeric proteins (Chimeras 1-3). As indicated to the right, a protein contains repressor activity (+) or has no repressor activity (–) based on its ability to suppress dorsoanterior structure formation, to repress the *siamois* luciferase reporter gene (S01234), and to repress the endogenous *siamois* gene following dorsal injections. HMG, the HMG Box DNA-binding domain; β, the β-catenin-binding domain. (B) XTcf-3 and Chimera 3 ventralize *Xenopus* embryos, but LEF-1 does not. Uninjected *Xenopus* embryos or embryos injected into the dorsal equatorial region at the 4-cell stage with 0.5 ng of *LEF-1*, *XTcf-3* or *Chimera 3* RNA were cultured to the tadpole stage and scored for dorsoanterior structure formation. (a) Control uninjected tadpole. (b) *LEF-1*-injected tadpoles are indistinguishable from controls. (c) *XTcf-3* injection results in severely ventralized embryos. (d) *Chimera 3* injection results in ventralized embryos, although they retain rudimentary dorsoanterior structures. (C) Chimeras 1, 2 and 3, like XTcf-3, repress the *siamois* reporter gene, but LEF-1 does not. The wild-type *siamois* reporter (S01234) was injected into the dorsal equatorial region of 4-cell stage *Xenopus* embryos either alone (control) or in the presence of RNA encoding the indicated proteins. Three pools of five stage 10 embryos each were assayed, and the mean and standard error of the resulting luciferase activities, expressed in relative lights units (RLUs), are shown. The average fold reduction in luciferase activity relative to the S01234 control is shown *above* each data set. N.S., no significant reduction in activity.

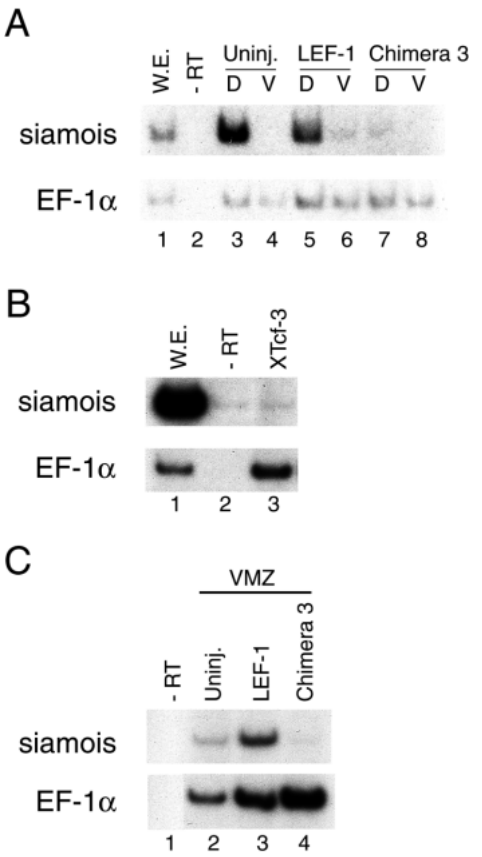

Fig. 2. The XTcf-3 C-terminal domain fused to LEF-1 (Chimera 3) has repressor activity, but LEF-1 does not. (A) Dorsal expression of the endogenous *siamois* gene is repressed by Chimera 3, but not by LEF-1. 4-cell stage embryos were injected at the equator of each dorsal or ventral blastomere with *Chimera 3* or *LEF-1* RNA. Dorsal or ventral marginal zones were explanted at stage 10 and the levels of endogenous *siamois* transcripts were measured by RT-PCR. Whole embryos (W.E.; lane 1); no reverse transcriptase (–RT; lane 2,) control; dorsal marginal zone (D) explants from uninjected (lane 3), *LEF-1* RNA-injected (lane 5), and *Chimera 3* RNA-injected (lane 7) embryos; ventral marginal zone (V) explants from uninjected (lane 4), *LEF-1* RNA-injected (lane 6), and *Chimera 3* RNA-injected (lane 8) embryos. (B) Endogenous *siamois* expression is repressed by XTcf-3. 4-cell stage embryos were injected at the equator of each blastomere with *XTcf-3* RNA and levels of endogenous *siamois* transcripts were measured by RT-PCR at stage 10. W.E. (lane 1), –RT (lane 2) and *XTcf-3* RNA-injected (lane 3). (C) Ventral injection of LEF-1 upregulates ventral *siamois* expression, while injection of Chimera 3 does not. The ventral marginal zone (VMZ) explants from A were subjected to additional cycles of PCR to detect ventral expression of *siamois*. –RT (lane 1), uninjected (lane 2), *LEF-1* RNA-injected (lane 3), and *Chimera 3* RNA-injected (lane 4).

RNA effectively ventralized embryos (Fig. 1B.c, compare to control, a; DAI=1.2, *n*=60), while embryos dorsally injected with 0.5 ng of *LEF-1* RNA were virtually indistinguishable from controls (Fig. 1B.b, compare to control, a; DAI=4.7, *n*=98). Similarly, *XTcf-3* RNA dorsally co-injected with the *siamois* reporter gene repressed its normal activity 28-fold, while *LEF-1* RNA co-injection had no significant effect on *siamois* reporter activity (Fig. 1C). In agreement with this finding, injected *XTcf-3* RNA eliminated endogenous *siamois* transcripts (Fig. 2B; compare lanes 1 and 3), whereas *LEF-1* injected embryos had *siamois* levels nearly identical to uninjected control embryos (Fig. 2A; compare lanes 3 and 5). The *LEF-1* RNA used for these assays was active since it induced a partial second axis in greater than 90% of ventrally injected embryos (data not shown), which is in agreement with a previous report (Behrens et al., 1996). These results support the proposal that XTcf-3 has a transcriptional repressor activity that LEF-1 lacks.

XTcf-3 contains N- and C-terminal repressor domains

Since XTcf-3 and LEF-1 have nearly identical HMG box DNA binding domains and bind the same DNA element, we reasoned that the repressor activity of XTcf-3 would be located outside this region. To test this, we constructed chimeras between LEF-1 and XTcf-3 (Fig. 1A). To our surprise, each chimera acted as a repressor based on the three assays described above (summarized in Fig. 1A). Synthetic RNAs encoding Chimeras 1, 2, and 3 ventralized embryos when injected into the dorsal equatorial region (DAI=1.3, *n*=26, 2.2, *n*=24, and 2.7, *n*=82, respectively), and when dorsally co-injected with the *siamois* reporter repressed its activity 23-fold, 12-fold and 9-fold, respectively (Fig. 1C). Moreover, a XTcf-3 construct lacking all residues of the C-terminal region (ΔC–XTcf-3) potently repressed dorsal axis formation and *siamois* expression (Fig. 1A). We hypothesized that both the N- and C-terminal regions of XTcf-3 contain repressor domains. The presence of a repressor domain in the N-terminal region is in agreement with a recent report demonstrating that a *Xenopus* homologue of the co-repressor Groucho binds this region of XTcf-3 (Roose et al., 1998).

We chose to focus our attention on the C-terminal domain of XTcf-3, as this region is absent from LEF-1 (Fig. 1A). While the C terminus is a somewhat less potent repressor than the N terminus, it still potently repressed dorsal axis formation (Fig. 1B.d, compare to control, Fig. 1B.a), *siamois* reporter activity (Fig. 1C) and endogenous *siamois* when dorsally expressed (Fig. 2A; compare lanes 3 and 7). In addition, unlike LEF-1, the C-terminus of XTcf-3 did not activate endogenous *siamois* when ectopically expressed ventrally (Fig. 2C; compare lanes 3 and 4).

XTcf-3 interacts with the transcriptional co-repressor *Xenopus* CtBP

To identify additional XTcf-3 binding proteins we performed a two-hybrid screen using XTcf-3 as bait, with a library of *Xenopus laevis* oocyte cDNAs. We identified one apparently full-length cDNA containing an open reading frame encoding a 440 amino acid polypeptide with extensive homology to the transcriptional co-repressor CtBP (Fig. 3). The predicted polypeptide shares 79% and 80% amino acid identity with the *Xenopus* (Sewalt et al., 1999) and human (Schaeper et al., 1995) CtBP1 proteins, respectively and 67% identity with *Drosophila* CtBP (Nibu et al., 1998b; Poortinga et al., 1998). While XCtBP1 is clearly the homologue of the mouse and human CtBP1 proteins (Sewalt et al., 1999), the relationship of the predicted polypeptide we have identified to CtBP1 and CtBP2 is unclear. XCtBP1 shares 95% and 80% amino acid identity with the mouse and human CtBP1 and CtBP2 proteins, respectively, whereas the protein we have identifed shares 80%

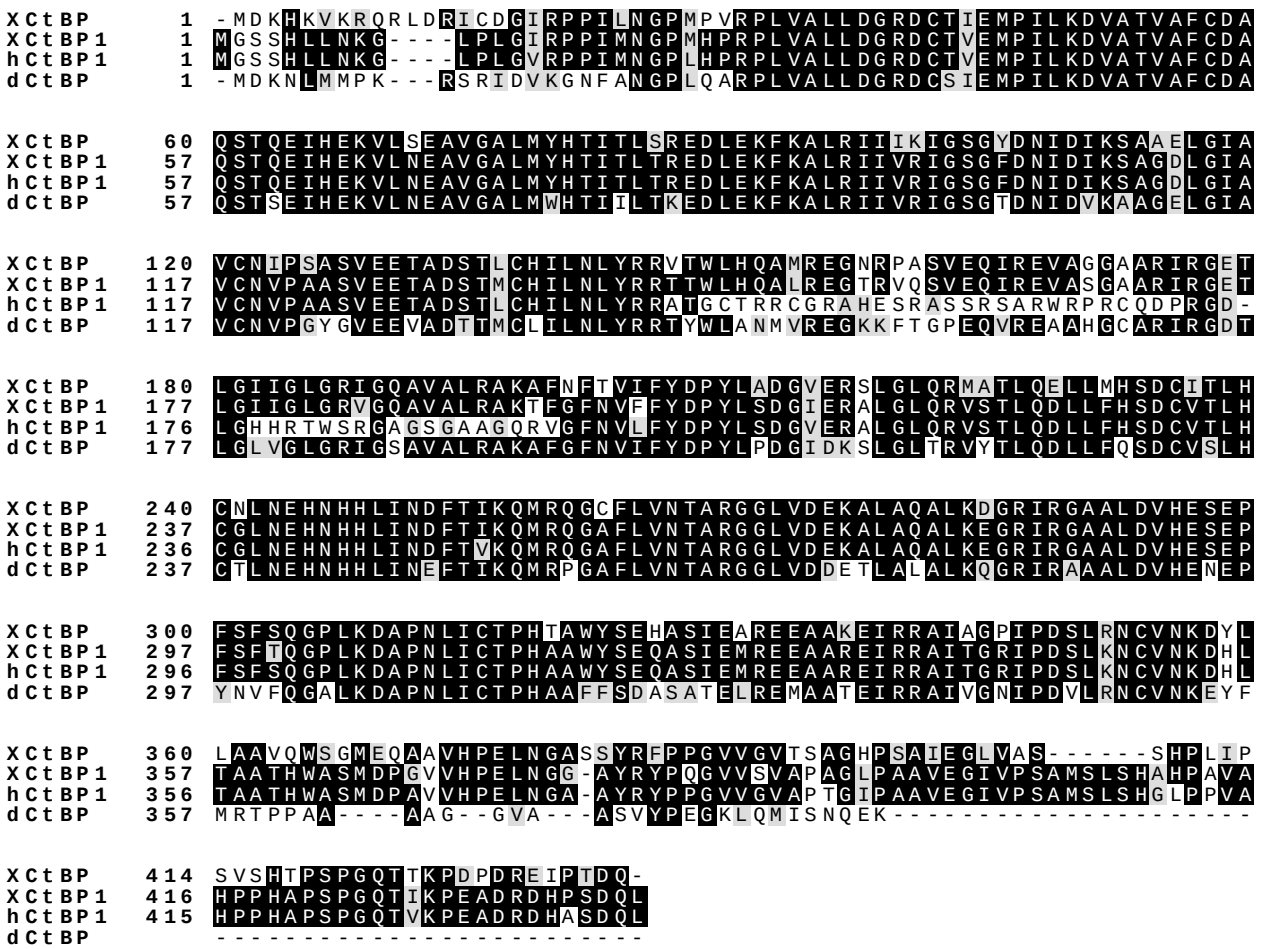

Fig. 3. Amino acid sequence of *Xenopus* CtBP compared with *Xenopus* and human CtBP1 and *Drosophila* CtBP. The *Xenopus* sequence derived from the original cDNA identified in the two-hybrid screen is shown. Amino acid identities are indicated by black boxes and similarities are indicated by gray boxes. Sequences were aligned with CLUSTALW and analyzed with BOXSHADE. The GenBank accession no. for XCtBP is AF152006.

and 76% identity with both forms of CtBP. Since this protein is not a CtBP1 and is not more likely, based on the amino acid identity, to be a CtBP2, we call this CtBP family member *Xenopus* CtBP (XCtBP; Fig. 3).

Temporal and spatial expression pattern of XCtBP

XCtBP is expressed throughout *Xenopus* development. Using RT-PCR, we find that maternal *XCtBP* transcripts are present before fertilization through the start of zygotic transcription (stage 8; Fig. 4A), but are not localized prior to the start of gastrulation (stage 10; Fig. 4B,C). *XCtBP* transcripts begin to accumulate with the onset of neurulation (stage 13), eventually peak in expression in the tailbud embryo (stage 27), and persist into the tadpole. The maternal expression of *XCtBP* indicates that it could function with maternal XTcf-3 to regulate *siamois* expression.

We determined the spatial expression pattern of *XCtBP* from gastrulation onward by in situ hybridization (Fig. 5A-K). At the beginning of gastrulation, as shown by RT-PCR (Fig. 4B,C), *XCtBP* transcripts are not spatially restricted (stage 10; Fig. 5A,B). Localized *XCtBP* transcripts first appear in a broad region of the anterior neural plate and in two stripes lateral to the midline at the early neurula stage (stage 13; Fig. 5C,D). Diffuse caudal *XCtBP* expression is also seen at stage 13 in the region of the future tailbud. At the end of neurulation, *XCtBP* transcripts are expressed in the eye placodes, along the branchial arches and in the developing brain (stage 20; Fig. 5E). This neural *XCtBP* expression extends the length of the embryo along the dorsal midline (Fig. 5F). Transverse sections of stage 20 embryos reveals that midline *XCtBP* transcripts are localized to the dorsal and lateral neural tube, a region of the embryo that also expresses two *Wnts*; *Wnt-1* and *Wnt-3a* (Wolda et al., 1993) (Fig. 5I). In this same view, *XCtBP* transcripts are also seen along the lateral surface of the somites. In dorsal and lateral views of a stage 20 embryo, *XCtBP* expression clearly outlines the somite borders and appears more intense in the tailbud (Fig. 5F,G). This pattern of somite and tailbud expression continues into the early tadpole (stage 33; Fig. 5J). *XCtBP* transcripts show continued expression throughout the head in early tadpoles, labeling the eyes, otic vesicles, branchial arches and brain, while more posteriorly *XCtBP* labels the pronephros (Fig. 5J). Curiously, *XCtBP* is excluded from the cement gland. Transverse sections of tadpoles show that *XCtBP* continues to be expressed in the dorsal and lateral neural tube (Fig. 5K). A sense control probe produced no signal (Fig. 5H).

CtBP binds to two conserved sites in the XTcf-3 C-terminal domain

CtBP interacts with transcription factors that contain a fairly well conserved amino acid motif (Turner and Crossley, 1998). We searched for this motif in XTcf-3 and found two sites in the XTcf-3 C-terminal region, PLSLT (site 1) and PLSLV (site 2), with the potential to bind XCtBP (Fig. 6). XCtBP site 1 is similar and site 2 is identical to the core CtBP recognition motif identified in the *Drosophila* repressor Hairy (Poortinga et al., 1998). In a BLAST search using the C-terminal sequence of XTcf-3, we determined that both sites are conserved in Tcf-3 homologues from zebrafish (Peleari and Maischein, 1998) and mouse (Korinek et al., 1998), as well as in the human Tcf-4 protein (Korinek et al., 1997) (Fig. 6). We do not find these sites in other Tcf family members, such as dTcf (Brunner et al., 1997; van de Wetering et al., 1997), Tcf-1 (van de Wetering et al., 1991) and POP-1 (Lin et al., 1995), nor do we find them outside of the Tcf-3 C-terminal domain.

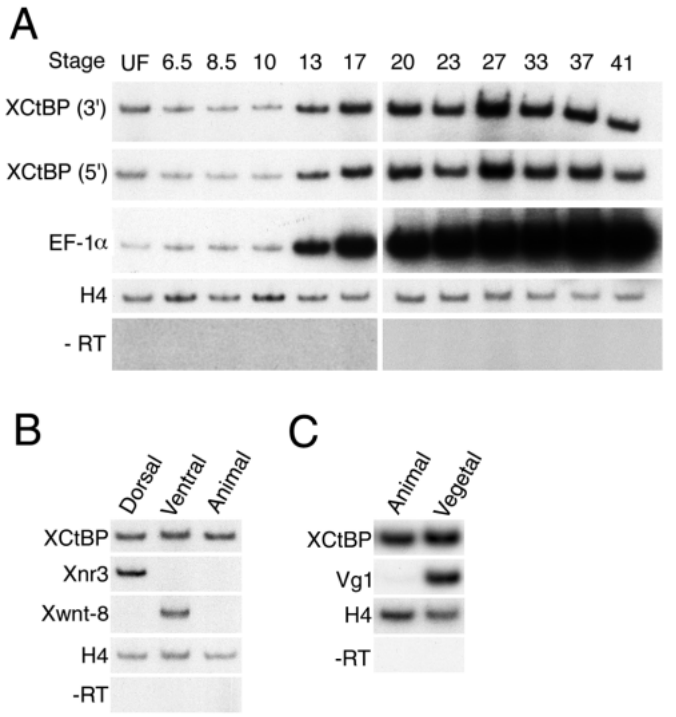

Fig. 4. Spatial and temporal expression of *XCtBP* during *Xenopus* development. (A) *XCtBP* is expressed at all stages of development. The temporal expression of *XCtBP* was analyzed by RT-PCR using oligonucleotide primer pairs directed against *XCtBP* 3′ and 5′ regions. *EF-1α* and *histone H4* are included as loading controls. The developmental stage analyzed is indicated above each lane; (UF) unfertilized eggs; (6.5) pre-midblastula transition (pre-zygotic transcription); (8.5) midblastula transition (start of zygotic transcription); (10) early gastrula; (13-20) early-late neurula; (27) tailbud; (33-41) early-late tadpole. (B) XCtBP is expressed at the same level in the dorsal, ventral and animal pole regions. Dorsal and ventral equatorial and animal pole tissues were dissected from stage 10 embryos and levels of *XCtBP* transcripts determined by RT-PCR. *Xnr3* and *Xwnt-8* mark dorsal and ventral tissues, respectively. (C) XCtBP is expressed equally in the animal and vegetal pole regions. Animal and vegetal pole tissues were dissected from stage 10 embryos and levels of *XCtBP* transcripts determined by RT-PCR. *Vg1* marks vegetal tissue.

To determine if these sites can bind CtBP, we performed a GST pull-down assay that was used previously to demonstrate the interaction between the *Drosophila* Hairy protein and *Drosophila* CtBP (dCtBP) (Poortinga et al., 1998). We used GST-dCtBP for convenience, since the *Xenopus* and *Drosophila* proteins are highly conserved (Fig. 3). ^{35}S-labeled Chimera 3 effectively bound to dCtBP (Fig. 7; lane 9). A previous report demonstrated that substituting Ala-Ser residues for the Pro-Leu residues in the CtBP core binding motif of the Adenovirus E1a protein eliminates binding to hCtBP (Schaeper et al., 1995). We introduced this mutation into site 1 or site 2 or both of the prospective XCtBP sites in the XTcf-3 portion of Chimera 3. The ability of GST-dCtBP to interact with Chimera 3 was lowered significantly when either XCtBP site was mutant (Fig. 7; lanes 10 and 11) and virtually abolished following mutation of both sites (Fig. 7; lane 12). These results indicate that CtBP binds XTcf-3 at both of the C-terminal XCtBP sites.

The XCtBP binding sites mediate the repressor activity of the XTcf-3 C-terminal domain

To evaluate the functional contribution of the XCtBP binding sites to XTcf-3-mediated transcriptional repression, we dorsally co-injected the normal and mutant forms of Chimera 3 together with the *siamois* reporter gene (S01234). We found it necessary to use Chimera 3 to analyze XCtBP activity since repression by the N-terminal Groucho-binding region of XTcf-3 masks any changes in repression caused by altering the C-terminal region. As described above, Chimera 3 represses the *siamois* reporter gene when they are co-injected into the dorsal equatorial region, resulting in an 8-fold decrease in activity (Fig. 8A). Chimera 3 mutant in either of the XCtBP binding sites was only slightly less effective than normal Chimera 3 at repressing the *siamois* reporter gene following dorsal co-injections, indicating that either XCtBP site retains repressor activity (data not shown). In contrast, the *siamois* reporter gene co-injected with Chimera 3 mutant in both XCtBP sites (Chimera 3/Double Mut.) or truncated to eliminate both sites (Chimera 3/Δ-CtBP) is nearly as active as the reporter gene injected alone, attaining activity levels 6- and 5-fold higher compared to co-injection with normal Chimera 3 (Fig. 8A). These results demonstrate that the XCtBP binding sites in the XTcf-3 C-terminus are critical to the repressor activity we have identified in this region. Moreover, these results are consistent with the observation that XCtBP is present as a maternal mRNA in *Xenopus* embryos, and indicate that it is translated to produce an active repressor.

Since *siamois* is only expressed dorsally (Lemaire et al., 1995), we previously proposed that the *siamois* gene is subject to XTcf-3-mediated repression everywhere in the embryo except the dorsal-vegetal region, where the XTcf-3 transcriptional co-activator β-catenin accumulates (Brannon et al., 1997). This led us to predict that a transcriptionally activating form of XCtBP would counter the repressing activity of XCtBP and activate ventral expression of *siamois*. We fused the GAL4 activation domain to XCtBP to produce XCtBP/G4A, and tested its effect on *siamois* reporter gene expression (Fig. 8B). While the *siamois* reporter gene injected alone was 20-fold more active following injection into the dorsal equatorial region when compared to ventral equatorial injections, ventral injections of the reporter gene in the

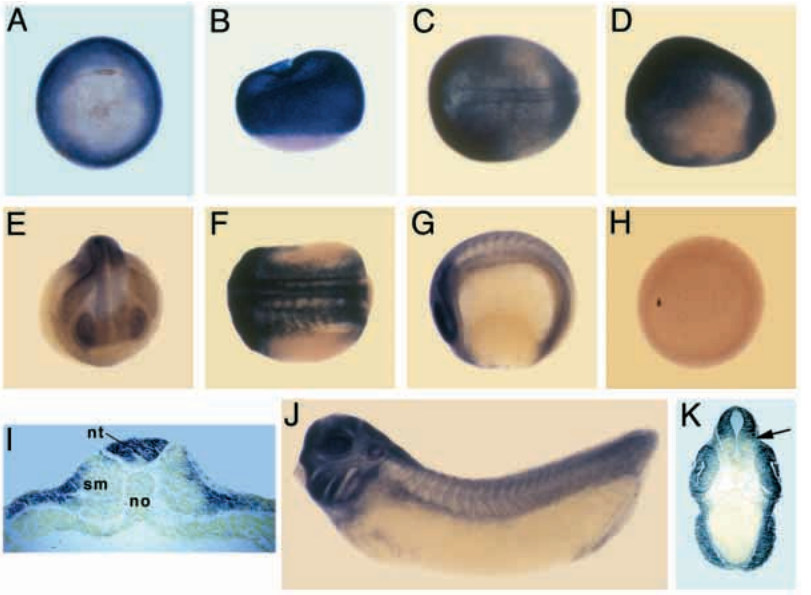

Fig. 5. Spatial expression pattern of *XCtBP* from the early gastrula to the tadpole stage. *XCtBP* expression was analyzed by whole-mount in situ hybridization using albinos, either cleared using Murray's clear (A-H,J) or sectioned (I,K). (A) Vegetal and (B) lateral views of a stage 10 embryo showing ubiquitous *XCtBP* expression. (C) Dorsal and (D) lateral views, with anterior to the left, of a stage 13 embryo showing *XCtBP* transcripts localized to the anterior neural plate, dorsal midline and future tailbud. (E) An anterior view, and (F) dorsal and (G) lateral views, with anterior to the left, of a stage 20 embryo. *XCtBP* transcripts can be seen in the neural and mesodermal tissues of the dorsal midline, in the tailbud and in the eyes, brain and branchial arches in the head. (H) No signal is observed in a stage 10 embryo hybridized with a sense control probe. (I) A transverse section posterior to the head of a stage 20 embryo, dorsal is at the top. *XCtBP* transcripts mark the dorsal neural tube (nt) and lateral somitic tissues, but are excluded from the notochord (no) and medial regions of the somites (sm). (J) Lateral view of a stage 33 embryo, anterior is to the left. *XCtBP* expression is intense in the head, labeling the eyes, otic vesicles, CNS and branchial arches. Posterior to the head, *XCtBP* is expressed in the pronephros, somites and tailbud. (K) A transverse section through the head of a stage 33 embryo, dorsal is at the top. *XCtBP* transcripts label the dorsal and lateral regions of the neural tube, but are excluded from the ventral neural tube (an arrow indicates the limit of *XCtBP* expression in the neural tube).

presence of *XCtBP/G4A* RNA resulted in a 35-fold increase in reporter activity (Fig. 8B). Ventral injection of *XCtBP/G4A* RNA also upregulated ventral expression of the endogenous *siamois* gene to a level similar to that induced by LEF-1 (Fig. 8C; compare lanes 6 and 8). There was only a minor increase in activity when *XCtBP/G4A* was ventrally co-injected with a mutant *siamois* reporter gene that no longer binds XTcf-3 (S24; Fig. 8D). These results demonstrate that XCtBP/G4A is a transcriptional activator that interacts with endogenous XTcf-3 to activate *siamois* gene expression, and thus acts antimorphically to endogenous XCtBP.

Antimorphic XCtBP reveals regulatory roles for XCtBP later in *Xenopus* development

While our data indicate that maternal XCtBP is involved in regulating *siamois* expression in the pre-gastrula embryo, the complex spatial pattern of *XCtBP* expression suggests that XCtBP also has roles during later stages of *Xenopus* development. In order to examine how XCtBP contributes to the development of the embryo, we injected *XCtBP* RNA at the 4-cell stage. Embryos dorsally overexpressing *XCtBP* were virtually indistinguishable from controls, suggesting that sufficient endogenous XCtBP is present during these stages to bind all potential protein partners (Fig. 9B, compare to control, A). Therefore, we performed similar injections with the transcriptionally activating XCtBP/G4A chimera, which acts at early stages as an antagonist of XCtBP (Fig. 8B, C). *XCtBP/G4A* affected all injected embryos (*n*=79), which were characterized by a loss of heads and/or eyes (82%), shortened anterior-posterior axes (97%), and mild spina bifida (53%); (Fig. 9C, compare to control, A). Importantly, co-injection of *XCtBP* with *XCtBP/G4A* restored normal development, demonstrating that the effect observed with XCtBP/G4A is specific (Fig. 9D, compare to control, A).

```
XTCF-3   412  METHTKT--------KKMCVQHLPADKSCDSPASSHGSMLDSPATPSAALASPAAPAATH
zTCF-3   420  SNFSPQP---------KKQCVPYLSSEKMCDSPTSSHGSMLDSPATPSAALASPAAPAATH
mTCF-3   441  GALASKS---------KKPCIQYLPPEKPCDSPASSHGSALDSPATPSAALASPAAPAATH
hTCF-4   445  FGLDQQNNWCGPCRRKKKCVRYIQGEGSCLSPPSSDGSLLDSPPPSPNLLGSPPRDAKSQ
LEF1     359  ASGTGPR----------MTAAYI-----------------------------------
                    1
XTCF-3   464  SEQAQPLSLTTKPEARAQLSLSHSAAFLASKSPSSSSFSGHLSSPVGSPLLSRPIPLTS-
zTCF-3   472  SEQAQPLSLTTKPEGRAHHNHPH------FPLPGKSSGSGSGSS-MALHSLSRPIPFTSL
mTCF-3   493  SEQAQPLSLTTKPEARAQLALHS---AAFLSAKAAASNSSQMGS--QPPLLSRPLPLGSM
hTCF-4   505  TEQTQPLSLSLKPDPLAHLSMMP----P-PPALLLAEATHKASALCPNGALDLPPAALQP
LEF1         ------------------------------------------------------------
                                                  2
XTCF-3   523  --SILSPSGVFPSALQALP---LLQAQPLSLVTKSSD
zTCF-3   525  PPSLLGPNSPFHQAALHSHHA-LLQTQPLSLVTKSVE
mTCF-3   548  PAALLTSPPTFPATLHAHQALPVLQAQPLSLVTKSAH
hTCF-4   560  AAPSSSIAQPSTSWLHSHSSLAGTQPQPLSLVTKSLE
LEF1         ---------------------------------------
```

Fig. 6. Amino acid sequence comparison of the C-terminal region of *Xenopus*, zebrafish and mouse Tcf-3, human Tcf-4 and human LEF-1. Amino acid identities are indicated by black boxes and amino acid similarities are indicated by gray boxes. Sequences were aligned with CLUSTALW and analyzed with BOXSHADE. Overlining indicates the two potential CtBP binding sites, which are labeled above each line (1, site 1 and 2, site 2).

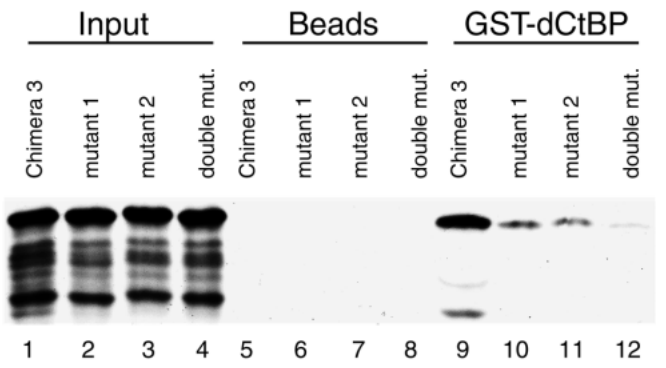

Fig. 7. In vitro interaction of the XTcf-3 C-terminal domain with CtBP. ^{35}S-labeled, full-length Chimera 3 protein (lane 1), binds to GST-dCtBP (lane 9). Chimera 3 proteins lacking CtBP binding site 1 (mutant 1; lane 2) or site 2 (mutant 2; lane 3), weakly interact with GST-dCtBP (lanes 10 and 11). Chimera 3 protein mutant in site 1 and site 2 (double mut.; lane 4) no longer efficiently binds to GST-dCtBP (lane 12). None of the Chimera 3 proteins bind to glutathione sepharose beads (beads; lanes 5-8) or to GST-bound beads (data not shown).

To analyze the XCtBP/G4A-mediated defects in more detail, we performed a histological examination of transverse-sectioned tadpoles (n=12). Most embryos (92%) have disorganized anterior neural tissue and at least one eye missing or reduced in size following injection of *XCtBP/G4A* (Fig. 9F, compare to control, E). *XCtBP/G4A* RNA injections also resulted in an absence or severe reduction of the notochord (50%), and in the majority of embryos that retained some notochord (75%), it was displaced lateral to the midline (Fig. 9H, compare to control, G).

In order to characterize more fully the XCtBP/G4A-mediated developmental defects we examined a number of genes that participate in early patterning by in situ hybridization. We found no significant change in the expression patterns of *goosecoid*, *chordin*, *Otx-2* or *MyoD* (data not shown). However, we observed that the expression of the early notochord marker *Xnot* was significantly reduced and disorganized (Fig. 9J, compare to control, I). These results suggest that endogenous XCtBP may participate in the regulation of *Xnot* expression and notochord development.

Since ectopic *XCtBP/G4A* activates ventral *siamois* expression (Fig. 8B,C), we initially expected that it would induce a second set of dorsoanterior structures, but this was not the case. Ventral overexpression of *XCtBP/G4A* resulted only in small, undifferentiated tissue outgrowths at the site of injection (data not shown). Taken together with the findings that XCtBP/G4A suppresses *Xnot* expression and notochord and head development, this result suggests that XCtBP also acts downstream of *siamois* to regulate later developmental processes.

DISCUSSION

The precise patterns of spatial and temporal gene expression observed throughout the development of complex organisms are in part generated by transcriptional repressor proteins that exert tight control over gene activation (Herschbach and Johnson, 1993; Gray and Levine, 1996; Ip and Hemavathy, 1997). A crucial regulatory function of Tcf transcription factors is to repress expression of target genes unless the transcriptional co-activator β-catenin/Armadillo is present (Clevers and van de Wetering, 1997; Kühl and Wedlich, 1997). As a consequence, mis-expression of β-catenin/Armadillo or loss of Tcf-mediated repression results in the inappropriate expression of these target genes. XTcf-3 and dTcf repress gene expression, in part, by recruiting the co-repressor Groucho (Cavallo et al., 1998; Roose et al., 1998). In *Drosophila*, the activation of dTcf is also repressed by CREB-binding protein, which inhibits the interaction of dTcf with the co-activator Armadillo by acetylating a specific lysine residue in the Armadillo-binding domain of dTcf (Waltzer and Bienz, 1998). Here we provide evidence that transcriptional repression by XTcf-3 is mediated by an additional co-repressor, XCtBP. We also find that XCtBP-mediated repression is involved in normal *Xenopus* development.

XCtBP is a XTcf-3 co-repressor

We have determined that the XTcf-3 N- and C-terminal domains both contain transcriptional repressor activity. When ectopically expressed in the dorsal equatorial region of early *Xenopus* embryos, XTcf-3 lacking the C-terminal domain (ΔC−XTcf-3) or chimeric proteins including the XTcf-3 N-terminal domain fused to hLEF-1 (Chimeras 1 and 2) block dorsoanterior structure formation and repress the activation of a *siamois* reporter gene and endogenous *siamois*. Repression by the XTcf-3 N-terminal domain is in accordance with the finding that this region of XTcf-3 binds Groucho (Roose et al., 1998). In this report we show that the XTcf-3 C-terminal domain is required for XCtBP-mediated repression. We demonstrate that the XCtBP binding sites are necessary and sufficient for XCtBP to bind XTcf-3 and for the repression mediated by the XTcf-3 C-terminal domain. XCtBP/G4A, a transcriptionally activating form of XCtBP, activates *siamois* expression, again indicating that endogenous XCtBP binds XTcf-3 and participates in the repression of XTcf-3 target genes. In addition to XTcf-3, we find that all known Tcf-3 homologues and hTcf-4 share the CtBP binding motif, suggesting an evolutionarily conserved CtBP repressor function for these Tcf family members.

Our studies make use of chimeras between hLEF-1 and the XTcf-3 C-terminal domain in order to avoid the repression we observed for the XTcf-3 Groucho binding domain. Contrary to a recently published report demonstrating that mLEF-1 interacts with the mammalian Groucho homologue TLE1 and represses a synthetic Tcf/Lef responsive gene (Levanon et al., 1998), we observe no repression using hLEF-1 in our assays. These authors propose that an amino acid motif in mLEF-1 (FRQPY), which is similar to a known Groucho interaction motif (WRPY) (Aronson et al., 1997), may mediate the interaction of mLEF-1 and TLE1. We note that this motif is absent in the hLEF-1 splice variant we used to make our chimeras.

The XCtBP and Groucho connection

While Tcf-3 is the first vertebrate protein to be shown to interact with both Groucho and CtBP, an intriguing parallel is found with the *Drosophila* Hairy protein. Hairy binds both Groucho and dCtBP, and genetic evidence indicates that both

co-repressors are important for Hairy function (Poortinga et al., 1998). Interestingly, Groucho and dCtBP function antagonistically with respect to Hairy, since removal of the Hairy dCtBP binding site enhances Groucho-mediated repression, whereas increasing the affinity between dCtBP and Hairy decreases Groucho-mediated repression (Zhang and Levine, 1999). This may be due to the close proximity of Hairy's Groucho and dCtBP binding sites, which are only nine amino acids apart, and raises the possibility that the binding of dCtBP and Groucho to Hairy is mutually exclusive. In contrast, the Groucho and XCtBP binding sites are well-separated in the primary sequence of XTcf-3, although it is possible that they could be close in the folded structure.

We have been unable to observe a clear effect on the repression of *siamois* by full-length XTcf-3 when the XCtBP sites are altered, indicating that Groucho plays a major role in the Tcf-3-dependent transcriptional repression of *siamois*. While it is possible that XCtBP is completely redundant with Groucho in early development, we note that *siamois* is a potent organizer of dorsoanterior structures and the combined activity of two XTcf-3 co-repressors may ensure that *siamois* is completely repressed on the ventral side of the embryo.

XCtBP in later development

It is likely that XCtBP has multiple roles in the development of the embryo. In *Xenopus*, we find a ubiquitous distribution of maternal transcripts that becomes localized to discrete structures during neurulation. The pattern of *XCtBP* expression is increasingly refined to include the head, central nervous system and tissues along the dorsal midline to the tailbud. Strikingly, the spatiotemporal expression pattern of *XCtBP* is mimicked by that of *XTcf-3* (Molenaar et al., 1998), suggesting that XCtBP and XTcf-3 share a functional relationship throughout development. *Wnts* have a role in the development of many of the structures to which *XCtBP* and *XTcf-3* co-localize (Moon et al., 1997). *En-2* is a well-studied *Wnt*-responsive gene that is localized to the midbrain-hindbrain boundary (McMahon et al., 1992), overlapping in expression with *Wnt-1* and *-3a* (Wolda et al., 1993). The *Xenopus En-2* promoter contains multiple Tcf/Lef consensus binding sites critical for both the Wnt-response and ΔN-XTcf-3-mediated repression of an *En-2* luciferase reporter gene (L. McGrew and R. T. M., unpublished results). These findings suggest that XCtBP may regulate additional *Wnt*-responsive genes in later development.

CtBP is likely to regulate *Wnt*-dependent genes in other contexts. As stated above, we find CtBP binding sites conserved in the known Tcf-3 homologues. Perhaps more interesting is the identification of CtBP binding sites in hTcf-4, the Tcf family member present in many colon tumor cell lines (Korinek et al., 1997). In human colon carcinoma and melanoma, mutations that inactivate APC or activate β-catenin result in constitutively

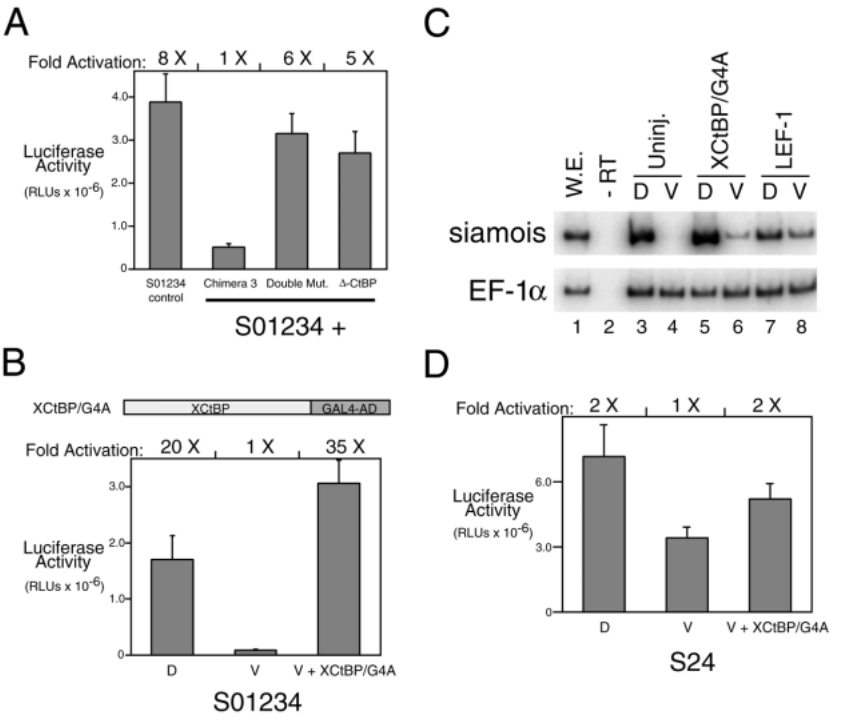

Fig. 8. The regulation of *siamois* expression by XCtBP. (A) The XTcf-3 C-terminal domain requires the XCtBP binding sites in order to repress *siamois* expression. The *siamois* luciferase reporter gene (S01234) was injected into the equatorial region of the dorsal blastomeres of 4-cell stage embryos either alone or in the presence of RNAs encoding Chimera 3, Chimera 3 mutated in both XCtBP binding sites (Double Mut.) or Chimera 3 truncated to remove both sites (Δ-CtBP). The mean and standard error of the resulting luciferase activities, in RLUs, from three pools of five embryos each are shown. The average fold activation of S01234 relative to the activity of S01234 repressed by Chimera 3 is shown *above* each data set. (B) XCtBP/G4A activates *siamois* expression. S01234 was injected at the 4-cell stage into the equatorial region of the dorsal blastomeres (D) or into the equatorial region of the ventral blastomeres (V) in the absence or presence of *XCtBP/G4A* RNA. The mean and standard error of the resulting luciferase activities, in RLUs, from three pools of five embryos each are shown. The average fold activation of S01234 relative to the activity of S01234 injected into the ventral blastomeres in the absence of *XCtBP/G4A* is shown *above* each data set. A diagram of XCtBP/G4A, which is full-length XCtBP with the GAL4 activation domain fused to its C terminus, is shown above the graph. (C) XCtBP/G4A activates ventral expression of endogenous *siamois*. 4-cell stage embryos were injected at the equator of each dorsal or ventral blastomere with *XCtBP/G4A* or *LEF-1* RNA. Dorsal or ventral marginal zones were explanted at stage 10 and the levels of endogenous *siamois* transcripts were measured by RT-PCR. Lane 1, whole embryos (W.E.); lane 2, no reverse transcriptase (-RT) control; dorsal marginal zone (D) explants from uninjected (lane 3), *XCtBP/G4A* RNA-injected (lane 5), and *LEF-1* RNA-injected (lane 7) embryos; ventral marginal zone (V) explants from uninjected (lane 4), *XCtBP/G4A* RNA-injected (lane 6), and *LEF-1* RNA-injected (lane 8) embryos. (D) The activation of *siamois* by XCtBP/G4A is mediated by XTcf-3. A *siamois* reporter gene with mutated Tcf/Lef binding sites that is unable to bind XTcf-3 (S24) was injected at the 4-cell stage into the equatorial region of the dorsal blastomeres (D) or into the equatorial region of the ventral blastomeres (V) in the absence or presence of *XCtBP/G4A* RNA. The mean and standard error of the resulting luciferase activities, in RLUs, from three pools of five embryos each are shown. The average fold activation of S24 relative to the activity of S24 injected into the ventral blastomeres in the absence of *XCtBP/G4A* is shown *above* each data set.

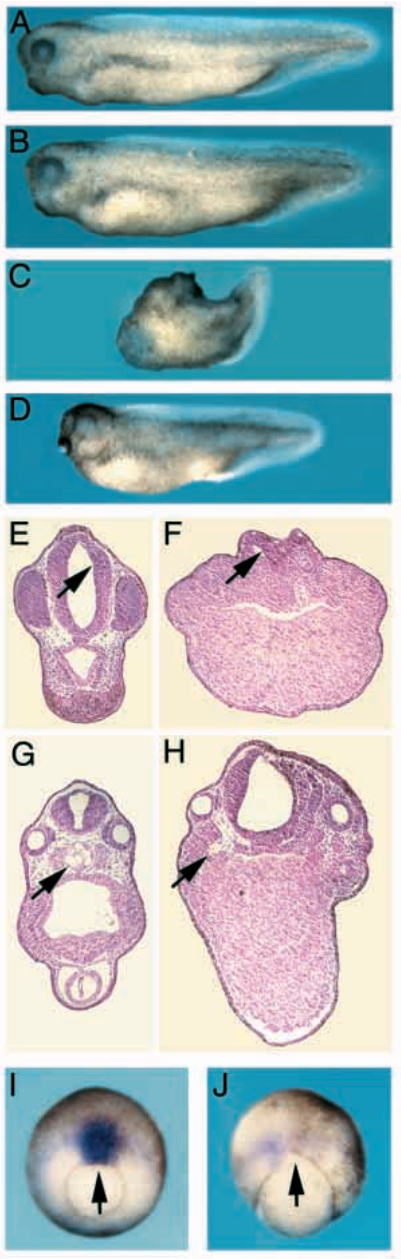

Fig. 9. Role of XCtBP in *Xenopus* development. (A-D) The XCtBP/G4A-mediated disruption of *Xenopus* development is XCtBP-specific. (A) A normal, uninjected control stage 35 embryo. (B) Embryos injected with XCtBP are indistinguishable from controls. A stage 35 embryo injected with 1 ng of *XCtBP* RNA at the equatorial region of the dorsal blastomeres at the 4-cell stage is shown. (C) XCtBP/G4A injections disrupt normal development, resulting in head loss and a shortened anterior-posterior axis. A stage 35 embryo similarly injected with 0.2 ng of *XCtBP/G4A* RNA. (D) XCtBP rescues all XCtBP/G4A-mediated developmental defects. A stage 35 embryo co-injected with 1 ng of *XCtBP* RNA and 0.2 ng of *XCtBP/G4A* RNA at the equatorial region of the dorsal blastomeres at the 4-cell stage is shown. (E-H) Histological examination of the XCtBP/G4A-mediated disruptions of *Xenopus* development. Transverse sections through the head of a control, uninjected stage 35 embryo at the position of the eyes (E) or the otic vesicles (G) (arrows indicate the neural tube and notochord). (F) A transverse section through the anterior portion of the head of an embryo injected with 0.2 ng of *XCtBP/G4A* RNA shows a major reduction or loss of all head structures, including the neural tube (arrow). (H) A transverse section through the head, at the level of the otic vesicles, of an embryo injected with 0.2 ng of *XCtBP/G4A* RNA shows a disorganization of the neural tube, a notochord (arrow) reduced in size and laterally displaced. Dorsal is at the top in all sections. (I-J) XCtBP/G4A blocks the expression of the notochord marker *Xnot*. Analysis of *Xnot* expression is by whole-mount in situ hybridization. (I) A control, uninjected stage 11.5 embryo shows the normal pattern of *Xnot* transcripts along the dorsal midline (arrow). (J) A stage 11.5 embryo injected with 0.2 ng of *XCtBP/G4A* RNA shows a severe reduction and disorganization of *Xnot* expression (arrow).

active Tcf-mediated transcription (Morin et al., 1997). Human CtBP1 is a known tumor suppressor which limits the transformation potential of the Adenovirus E1a oncoprotein (Subramanian et al., 1989). Importantly, CtBP1 and CtBP2 are differentially expressed in many human cancer cell lines (Sewalt et al., 1999).Therefore, it will be interesting to investigate whether CtBP is a hTcf-4 co-repressor, and to determine the status of CtBP in cancerous tissues that inappropriately activate hTcf-4.

To investigate the role of XCtBP in later *Xenopus* development, we ectopically expressed the antimorphic XCtBP, XCtBP/G4A. Co-injection of *XCtBP* reversed all *XCtBP/G4A*-mediated effects, demonstrating that they were specific to XCtBP. Overexpression of *XCtBP/G4A* primarily caused loss of the head, neural defects and a shortening of the anterior-posterior axis. The head and neural defects might be due to the mis-expression of posterior genes in the anterior region of the embryo, which is a known effect of *Wnt* mis-expression (Hoppler et al., 1996).

The shortened anterior-posterior axis is likely to be due to alterations in the notochord, which was seen primarily as an inhibition of the early notochord marker *Xnot* (Gont et al., 1993; von Dassow et al., 1993). This result may also be attributable to mimicking a *Wnt* effect, since ectopic *Wnt-8* expression also inhibits *Xnot*, while ectopic expression of a dominant negative *Wnt* mutant expands the *Xnot* expression domain (Hoppler et al., 1996). Our results differ from these, however, in that we did not observe a concomitant expansion of *MyoD* expression into the midline when *Xnot* was inhibited (unpublished results). This suggests the possibility of other XCtBP-dependent transcription factors expressed in the dorsal midline.

The proposal that XCtBP may participate in XTcf-3-independent processes later in development is further supported by the finding that although XCtBP/G4A activates the *siamois* reporter gene in a XTcf-3-dependent manner, it does not duplicate the axis when ventrally overexpressed. In contrast, ventral overexpression of β-catenin, which also activates *siamois* through an interaction with XTcf-3 (Brannon et al., 1997), causes a complete axis duplication (Funayama et al., 1995). If XTcf-3 and *siamois* were the only targets of XCtBP we would expect a similar axis duplication using XCtBP/G4A. As discussed above, XCtBP/G4A suppresses head and notochord formation when overexpressed on the dorsal side of *Xenopus* embryos, and therefore, the lack of dorsoanterior structures following ventral injections of *XCtBP/G4A* is not surprising. These results suggest that there are transcription factor targets of XCtBP, in addition to XTcf-3 and downstream of *siamois*, that participate in dorsoanterior

structure formation. Furthermore, XCtBP is also likely to regulate non-*Wnt* pathways, since CtBP has been shown to bind a number of transcription factors, including bHLH, zinc-finger and nuclear receptor family members (Nibu et al., 1998a; Turner and Crossley, 1998).

Conclusion

The evidence to date indicates that CtBP is a co-repressor for a variety of transcription factors. We have identified XCtBP and demonstrated that it serves as a co-repressor for XTcf-3 and probably other *Xenopus* transcription factors. We have identified a critical role for XCtBP in *Xenopus* development using XCtBP/G4A, a transcriptionally activating form of XCtBP. XCtBP/G4A should prove to be a useful tool in dissecting developmental processes mediated by XTcf-3 and other XCtBP binding proteins.

We are grateful to Susan Parkhurst for providing GST-dCtBP plasmid and GST protein, David Turner for CS2+ vectors, Marian Waterman for hLEF-1, Olivier Destrée for XTcf-3 and Taryn Phippen and Denise Ferkey for generously providing technical advice and assistance. We thank Anna E. Melby and Carole Weaver for critical comments on the manuscript. This work was supported by National Institutes of Health (NIH) Predoctoral Training Grants 5T32HDO7183-17 (to M. B.), and 2T32HDO7183-16 (to J. D. B.), and NIH grants HD27262 (to D. K.) and HD29360 (to R. T. M.). R. T. M. is an Investigator of the Howard Hughes Medical Institute.

REFERENCES

Aronson, B. D., Fisher, A. L., Blechman, K., Caudy, M. and Gergen, J. P. (1997). Groucho-dependent and -independent repression activities of Runt domain proteins. *Mol. Cell. Biol.* **17**, 5581-5587.

Behrens, J., von Kries, J. P., Kühl, M., Bruhn, L., Wedlich, D., Grosschedl, R. and Birchmeier, W. (1996). Functional interaction of β-catenin with the transcription factor LEF-1. *Nature* **382**, 638-642.

Bienz, M. (1998). TCF: transcriptional activator or repressor? *Curr. Opin. Cell Biol.* **10**, 366-372.

Brannon, M., Gomperts, M., Sumoy, L., Moon, R. T. and Kimelman, D. (1997). A β-catenin/XTcf-3 complex binds to the *siamois* promoter to regulate specification of the dorsal axis in *Xenopus*. *Genes Dev.* **11**, 2359-2370.

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

Brunner, E., Peter, O., Schweizer, L. and Basler, K. (1997). *pangolin* encodes a Lef-1 homologue that acts downstream of Armadillo to transduce the Wingless signal in *Drosophila*. *Nature* **385**, 829-833.

Cadigan, K. M. and Nusse, R. (1997). *Wnt* signaling: a common theme in animal development. *Genes Dev.* **11**, 3286-3305.

Cavallo, R. A., Cox, R. T., Moline, M. M., Roose, J., Polevoy, G. A., Clevers, H., Peifer, M. and Bejsovec, A. (1998). *Drosophila* Tcf and Groucho interact to repress Wingless signalling activity. *Nature* **395**, 604-608.

Clevers, H. and van de Wetering, M. (1997). TCF/LEF factors earn their wings. *Trends Genet.* **13**, 485-489.

Cox, R. T. and Peifer, M. (1998). *Wingless* signaling: the inconvenient complexities of life. *Curr. Biol.* **8**, R140-144.

Cui, Y., Brown, J. D., Moon, R. T. and Christian, J. L. (1995). *Xwnt8b*: A maternally expressed *Xenopus Wnt* gene with a potential role in establishing the dorso-ventral axis. *Development* **121**, 2177-2186.

Fan, M. J., Gruning, W., Walz, G. and Sokol, S. Y. (1998). Wnt signaling and transcriptional control of *Siamois* in *Xenopus* embryos. *Proc. Natl. Acad. Sci. USA* **95**, 5626-31.

Funayama, N., Fagotto, F., McCrea, P. and Gumbiner, B. M. (1995). Embryonic axis induction by the Armadillo repeat domain of β-catenin: evidence for intracellular signaling. *J. Cell Biol.* **128**, 959-968.

Gont, L. K., Steinbesser, H., Blumberg, B. and DeRobertis, E. M. (1993). Tail formation as a continuation of gastrulation: the multiple cell populations of the *Xenopus* tailbud derive from the late blastopore lip. *Development* **119**, 991-1004.

Gray, S. and Levine, M. (1996). Transcriptional repression in development. *Curr. Opin. Cell Biol.* **8**, 358-364.

Harland, R. M. (1991). *In situ* hybridization: an improved whole mount method for *Xenopus* embryos. In *Methods in Cell Biology* (ed. B. K. Kay and H. J. Peng) San Diego, Calif.: Academic Press Inc.

Hemmati-Brivanlou, A., Kelly, O. G. and Melton, D. A. (1994). Follistatin, an antagonist of activin, is expressed in the Spemann organizer and displays direct neuralizing activity. *Cell* **77**, 283-295.

Herschbach, B. M. and Johnson, A. D. (1993). Transcriptional repression in eukaryotes. *Annu. Rev. Cell Biol.* **9**, 479-509.

Hoppler, S., Brown, J. D. and Moon, R. T. (1996). Expression of a dominant negative *Wnt* blocks induction of *MyoD* in *Xenopus* embryos. *Genes Dev.* **10**, 2805-2817.

Huber, O., Korn, R., McLaughlin, J., Ohsugi, M., Herrmann, B. G. and Kemler, R. (1996). Nuclear localization of β-catenin by interaction with transcription factor LEF-1. *Mech. Dev.* **59**, 3-10.

Ip, Y. T. and Hemavathy, K. (1997). *Drosophila* development. Delimiting patterns by repression. *Curr. Biol.* **7**, R216-218.

Kao, K. R. and Elinson, R. P. (1988). The entire mesodermal mantle behaves as Spemann's organizer in dorsoanterior enhanced *Xenopus laevis* embryos. *Dev. Biol.* **127**, 64-77.

Kelly, G. M., Eib, D. W. and Moon, R. T. (1991). Histological preparation of *Xenopus laevis* oocytes and embryos. *Methods Cell Biol.* **36**, 389-417.

Korinek, V., Barker, N., Morin, P. J., van Wichen, D., de Weger, R., Kinzler, K. W., Vogelstein, B. and Clevers, H. (1997). Constitutive transcriptional activation by a β-catenin-Tcf complex in *APC–/–* colon carcinoma. *Science* **275**, 1784-1787.

Korinek, V., Barker, N., Willert, K., Molenaar, M., Roose, J., Wagenaar, G., Markman, M., Lamers, W., Destree, O. and Clevers, H. (1998). Two members of the Tcf family implicated in Wnt/β-catenin signaling during embryogenesis in the mouse. *Mol. Cell. Biol.* **18**, 1248-1256.

Kühl, M. and Wedlich, D. (1997). *Wnt* signalling goes nuclear. *BioEssays* **19**, 101-104.

Kunkel, T. A., Roberts, J. D. and Zakour, R. A. (1987). Rapid and efficient site-specific mutagenesis without phenotypic selection. *Methods Enzymol.* **154**, 367-382.

Larabell, C. A., Rowning, B. A., Wells, J., Wu, M. and Gerhart, J. (1996). Confocal microscopy of living *Xenopus* eggs and the mechanism of cortical rotation. *Development* **122**, 1281-1289.

Laurent, M. N., Blitz, I. L., Hashimoto, C., Rothbächer, 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.

Lemaire, P., Garrett, N. and Gurdon, J. B. (1995). Expression cloning of *Siamois*, a *Xenopus* homeobox gene expressed in dorsal-vegetal cells of blastulae and able to induce a complete secondary axis. *Cell* **81**, 85-94.

Levanon, D., Goldstein, R. E., Bernstein, Y., Tang, H., Goldenberg, D., Stifani, S., Paroush, Z. and Groner, Y. (1998). Transcriptional repression by AML1 and LEF-1 is mediated by the TLE/Groucho corepressors. *Proc. Natl. Acad. Sci. USA* **95**, 11590-11595.

Lin, R., Thompson, S. and Priess, J. R. (1995). *pop-1* encodes an HMG box protein required for the specification of a mesoderm precursor in early *C. elegans* embryos. *Cell* **83**, 599-609.

McKendry, R., Hsu, S. C., Harland, R. M. and Grosschedl, R. (1997). LEF-1/TCF proteins mediate Wnt-inducible transcription from the *Xenopus* nodal-related 3 promoter. *Dev. Biol.* **192**, 420-431.

McMahon, A. P., Joyner, A. L., Bradley, A. and McMahon, J. A. (1992). The midbrain-hindbrain phenotype of *Wnt-1-/Wnt-1-* mice results from stepwise deletion of *engrailed*-expressing cells by 9.5 days postcoitum. *Cell* **69**, 581-595.

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

Molenaar, M., van de Wetering, M., Oosterwegel, M., Peterson-Maduro, J., Godsave, S., Korinek, V., Roose, J., Destrée, O. and Clevers, H. (1996). XTcf-3 transcription factor mediates β-catenin-induced axis formation in *Xenopus* embryos. *Cell* **86**, 391-399.

Moon, R. T., Brown, J. D. and Torres, M. (1997). *WNTs* modulate cell fate and behavior during vertebrate development. *Trends Genet.* **13**, 157-162.

Moon, R. T. and Kimelman, D. (1998). From cortical rotation to organizer

gene expression: toward a molecular explanation of axis specification in *Xenopus*. *BioEssays* **20**, 536-545.

Morin, P. J., Sparks, A. B., Korinek, V., Barker, N., Clevers, H., Vogelstein, B. and Kinzler, K. W. (1997). Activation of β-catenin-Tcf signaling in colon cancer by mutations in β-catenin or APC. *Science* **275**, 1787-1790.

Nibu, Y., Zhang, H., Bajor, E., Barolo, S., Small, S. and Levine, M. (1998a). dCtBP mediates transcriptional repression by knirps, Krüppel and snail in the *Drosophila* embryo. *EMBO J.* **17**, 7009-7020.

Nibu, Y., Zhang, H. and Levine, M. (1998b). Interaction of short-range repressors with *Drosophila* CtBP in the embryo. *Science* **280**, 101-104.

Niehrs, C., Steinbesser, H. and De Robertis, E. M. (1994). Mesodermal patterning by a gradient of the vertebrate homeobox gene *goosecoid*. *Science* **263**, 817-820.

Nieuwkoop, P. D. and Faber, J. (1967). Normal table of *Xenopus laevis*. Amsterdam: North-Holland Publishing Company.

Peleari, F. and Maischein, H. M. (1998). Function of zebrafish β-catenin and TCF-3 in dorsoventral patterning. *Mech. Dev.* **77**, 63-74.

Pierce, S. B. and Kimelman, D. (1995). Regulation of Spemann organizer formation by the intracellular kinase Xgsk-3. *Development* **121**, 755-765.

Poortinga, G., Watanabe, M. and Parkhurst, S. M. (1998). *Drosophila* CtBP: a Hairy-interacting protein required for embryonic segmentation and hairy-mediated transcriptional repression. *EMBO J.* **17**, 2067-2078.

Riese, J., Yu, X., Munnerlyn, A., Eresh, S., Hsu, S.-C., Grosschedl, R. and Bienz, M. (1997). LEF-1, a nuclear factor coordinating signalling inputs from *wingless* and *decapentaplegic*. *Cell* **88**, 777-787.

Rocheleau, C. E., Downs, W. D., Lin, R., Wittmann, C., Bei, Y., Cha, Y. H., Ali, M., Priess, J. R. and Mello, C. C. (1997). Wnt signaling and an APC-related gene specify endoderm in early *C. elegans* embryos. *Cell* **90**, 707-716.

Roose, J., Molenaar, M., Peterson, J., Hurenkamp, J., Brantjes, H., Moerer, P., van de Wetering, M., Destree, O. and Clevers, H. (1998). The *Xenopus* Wnt effector XTcf-3 interacts with Groucho-related transcriptional repressors. *Nature* **395**, 608-612.

Schaeper, U., Boyd, J. M., Verma, S., Uhlmann, E., Subramanian, T. and Chinnadurai, G. (1995). Molecular cloning and characterization of a cellular phosphoprotein that interacts with a conserved C-terminal domain of adenovirus E1A involved in negative modulation of oncogenic transformation. *Proc. Natl. Acad. Sci. USA* **92**, 10467-10471.

Schmidt, J. E., von Dassow, G. and Kimelman, D. (1996). Regulation of dorsal-ventral patterning: the ventralizing effects of the novel *Xenopus* homeobox gene *Vox*. *Development* **122**, 1711-1721.

Schneider, S., Steinbeisser, H., Warga, R. M. and Hausen, P. (1996). β-catenin translocation into nuclei demarcates the dorsalizing centers in frog and fish embryos. *Mech. Dev.* **57**, 191-198.

Sewalt, R. G., Gunster, M. J., van der Vlag, J., Satijn, D. P. and Otte, A. P. (1999). C-terminal Binding Protein is a transcriptional repressor that interacts with a specific class of vertebrate Polycomb proteins. *Mol. Cell. Biol.* **19**, 777-787.

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

Subramanian, T., La Regina, M. and Chinnadurai, G. (1989). Enhanced ras oncogene mediated cell transformation and tumorigenesis by adenovirus 2 mutants lacking the C-terminal region of E1a protein. *Oncogene* **4**, 415-420.

Thorpe, C. J., Schlesinger, A., Carter, J. C. and Bowerman, B. (1997). Wnt signaling polarizes an early *C. elegans* blastomere to distinguish endoderm from mesoderm. *Cell* **90**, 695-705.

Turner, D. L. and Weintraub, H. (1994). Expression of *achaete-scute* homolog 3 in *Xenopus* embryos converts ectodermal cells to a neural fate. *Genes Dev.* **8**, 1434-1447.

Turner, J. and Crossley, M. (1998). Cloning and characterization of mCtBP2, a co-repressor that associates with basic Krüppel-like factor and other mammalian transcriptional regulators. *EMBO J.* **17**, 5129-5140.

van de Wetering, M., Cavallo, R., Dooijes, D., van Beest, M., van Es, J., Loureiro, J., Ypma, A., Hursh, D., Jones, T., Bejsovec, A., Peifer, M., Mortin, M. and Clevers, H. (1997). Armadillo co-activates transcription driven by the product of the *Drosophila* segment polarity gene *dTCF*. *Cell* **88**, 789-799.

van de Wetering, M., Oosterwegel, M., Dooijes, D. and Clevers, H. (1991). Identification and cloning of TCF-1, a T lymphocyte-specific transcription factor containing a sequence specific HMG box. *EMBO J.* **10**, 123-132.

von Dassow, G., Schmidt, J. E. and Kimelman, D. (1993). Induction of the *Xenopus* organizer: expression and regulation of *Xnot*, a novel FGF and activin-regulated homeobox gene. *Genes Dev.* **7**, 355-366.

Waltzer, L. and Bienz, M. (1998). *Drosophila* CBP represses the transcription factor TCF to antagonize *Wingless* signalling. *Nature* **395**, 521-525.

Wodarz, A. and Nusse, R. (1998). Mechanisms of *Wnt* signaling in development. *Annu. Rev. Cell Dev. Biol.* **14**, 59-88.

Wolda, S. L., Moody, C. J. and Moon, R. T. (1993). Overlapping expression of *Xwnt-3A* and *Xwnt-1* in neural tissue of *Xenopus laevis* embryos. *Dev. Biol.* **155**, 46-57.

Yang-Snyder, J., Miller, J. R., Brown, J. D., Lai, C. and Moon, R. T. (1996). A *frizzled* homolog functions in a vertebrate *Wnt* signaling pathway. *Curr. Biol.* **6**, 302-306.

Zhang, H. and Levine, M. (1999). Groucho and dCtBP mediate separate pathways of transcriptional repression in the *Drosophila* embryo. *Proc. Natl. Acad. Sci. USA* **96**, 535-540.