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

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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; Brunnert 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 repress siamois (Brannon et al., 1997).
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 XCIIBP as a protein that binds to 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 XCIIBP and used it to demonstrate the in vivo interaction between XCIIBP and XTcf-3 in the regulation of siamois. Antimorphic XCIIBP also reveals additional roles for XCIIBP-mediated repression at later stages of development.

MATERIALS AND METHODS

Cloning of XCIIBP

An amplified Xenopus laevis oocyte library in the vector pGAD10 (Clontech) was screened with full-length XTcf-3 cloned into pGBl9 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 XCIIBP was identified among the putative interacting clones.

DNA constructs

The XCtBP clone was excised from pGAD10 by restriction with SalI, cloned into the Xhol site of CS2+ (CS2+ was a gift from R. Davis) to produce XCIIBP-CS2+. To fuse the GAL4 activation domain in frame to the C terminus of XCtBP, XCIIBP was amplified by PCR using the CS2+ SP6 primer and oligonucleotide MB27 (5′-GGAG-3′) and MB11 (5′-GA TGC-3′) and MB12 (5′-GTGCCTGCAGCAG-3′) primer to amplify Chimera 3 by PCR. The amplification product was directly cloned into EcoRI and Xbal digested CS2+ to produce Chimera 1, 2 and 3.

Chimera 3 mutants that lack XCIIBP binding sites were produced as follows. Chimera 3 C-terminally truncated at XCIIBP site 1 (Chimera 3/ΔCtBP) was generated using oligonucleotide MB21 (5′-GGCTG- TAGATCAGGTTGCTCAGAGTGAG-3′), which introduces a Xbal site (underlined) and a termination codon (in bold), with the SP6 primer to amplify Chimera 3 by PCR. The amplification product was directly cloned into EcoRI and Xbal digested CS2+ to give Chimera 3/ΔCtBP-CS2+. To produce Chimera 3 mutant in XCIIBP site 2, oligonucleotide MB24 (5′-GGCTGACTAGTCAGCTCAGTGATT- TGGTACGAGAGACAGGCTTGTGCAGCAGCAG-3′), which introduces Xbal and SfiI 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 EcoRI and Xbal digested CS2+ to produce Chimera 3 mutant 2-2 CS2+. Oligonucleotide-mediated, site-directed mutagenesis (Kunkel et al., 1987) was used to produce the Chimera 3 XCIIBP site 1 and double mutants. The mismatched oligonucleotide MB25 (5′-CTCTAGAGCTAAGAGGTGAGGCTCTGCCCTCGACACCA-3′), which introduces a SfiI 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 NotI 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 XCIIBP/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 described: EF-1α (Hemmatti-Brivanlou et al., 1994), histone H4 (Niehrs et al., 1994), Xenot-8 and Vg1 (Cui et al., 1995), Xnr-3 and siamios (Yang-Snyder et al., 1996). Primers to the 5′-region of XCIIBP were, forward (5′-CAGAGAGTTTCTCAGGAGG-3′) and reverse (5′-CAGTAG- CTCTAGAGGCTCAGGCCC-3′) and primers to the 3′-region of XCIIBP were, forward (5′-GCTGCGCTTACCTCGACAC-3′) and reverse (5′-TACAGGTTCTGCTGTTTCC-3′).

Full digestion assays using GST-dCIIBP and 35S-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 BamHI and transcribed with T7 RNA polymerase to produce the antisense probe and XhoI 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.](image)

**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.
transcripts (Fig. 2B; compare lanes 1 and 3), whereas LEF-1 injected embryos had siamois levels nearly identical to un.injected 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 (∆C–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 identified shares 80%
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., 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 (Poortinga et al., 1998). We used GST-dCtBP for convenience, since the Xenopus and Drosophila proteins are highly conserved (Fig. 3). 35S-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).

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 (SO1234). We found that the normal and mutant Chimera 3 repressors are active, that the mutant Chimera 3 repressors are not active, and that the mutant Chimera 3 repressors are active.

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) 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 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).

Antimorphic XCtBP reveals regulatory roles for XCtBP later in Xenopus development

While our data indicate that maternal XCtBP is involved in regulating stamois 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).

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).
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 (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 mutant; 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).

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 (AC–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 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 for these Tcf family members.

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**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 AN-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.

XCtBP is likely to regulate Wnt-dependent genes in other contexts. As stated above, we find XCtBP 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

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### 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 (A-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.
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 antithomorph 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 CIBP 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 CIBP 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.

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