



xBtg-x regulates Wnt/ β -Catenin signaling during early *Xenopus* development

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Abstract

In *Xenopus*, two signaling systems, maternal β -Catenin and Nodal-related, are required for induction of the Spemann organizer and establishment of the body plan. By screening cDNA macroarrays for genes activated by these two signaling pathways, we identified *Xenopus xBtg-x*, a novel member of the Btg/Tob gene family of antiproliferative proteins. We show that *xBtg-x* is expressed in the dorsal mesendoderm (Spemann organizer tissue) of gastrula stage embryos and that its expression is regulated by both β -Catenin and Nodal-related signals. Microinjection of synthetic *xBtg-x* mRNA into *Xenopus* embryos induced axis duplication and completely rescued the ventralizing effects of UV irradiation through the activation of the canonical Wnt/ β -Catenin signaling pathway. Interestingly, *xBtg-x* stimulated β -Catenin-dependent transcription without affecting the stability of β -Catenin protein. These data suggest that *xBtg-x* is a novel component of the Wnt/ β -Catenin signaling pathway regulating early embryonic patterning.

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Introduction

In amphibians, the dorsal blastopore lip of the gastrula stage embryo, or Spemann organizer, determines the dorso-ventral body axis. When transplanted into the ventral side, this group of cells induces the formation of a secondary body, including a central nervous system (CNS), dorsal mesoderm, and a secondary gut (Spemann and Mangold, 1924; De Robertis and Kuroda, 2004). The specification of the Spemann organizer is initiated during fertilization when sperm entry triggers the assembly of a microtubule network resulting in a cortical rotation of the cytoplasm in the fertilized egg. Subsequently, maternal β -Catenin protein becomes stabilized on the future dorsal side of the embryo resulting in its nuclear localization (Schneider et al., 1996; Larabell et al., 1997) and the induction of β -Catenin target

genes such as *Xnr-3*, *siamois*, and *chordin* (Wessely et al., 2001). Though it is still unclear whether this process requires an endogenous Wnt molecule, this paradigm of axis formation has been instrumental for the dissection of the Wnt/ β -Catenin signaling pathway (Wodarz and Nusse, 1998). Microinjection of synthetic mRNAs encoding components of the Wnt/ β -Catenin signaling pathway such as *Wnt-1* or β -Catenin into the ventral side of the *Xenopus* embryo induces a secondary body axis mimicking the activity of Spemann organizer. Conversely, inhibition of the early β -Catenin signal by ultraviolet (UV) irradiation of fertilized eggs (which inhibits cortical rotation and nuclear localization of β -Catenin), or *Gsk-3 β Axin* or a dominant-repressive version of the transcription factor *XTcf-3* (ΔN -*XTcf-3*) abolishes dorsal development (Heasman, 1997; De Robertis et al., 2000).

Over the past few years, it has become evident that the initiation of dorsal development involves crosstalk with other signaling pathways in addition to the canonical Wnt/

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β -Catenin signaling. For example, non-canonical Wnt/ Ca^{2+} signaling has been shown to regulate ventral cell fate decisions by antagonizing the dorsal Wnt/ β -Catenin pathway. Inhibition of Wnt/ Ca^{2+} signaling in *Xenopus* by microinjection of a dominant-negative *calcium-calmodulin-dependent protein kinase II* (*CamKII*) or dominant-negative *Wnt-11* or by modulating intracellular Ca^{2+} levels promotes dorsal development (Kume et al., 1997; Kuhl et al., 2000; Westfall et al., 2003b). Similarly, zebrafish mutant embryos lacking the maternal and zygotic function of non-canonical Wnt-5 signals are dorsalized, have increased levels of nuclear β -Catenin, and ectopically express the β -Catenin target gene *bozozok*, suggesting an intersection between the canonical Wnt/ β -Catenin and the Wnt/ Ca^{2+} signaling pathways at the level of β -Catenin (Westfall et al., 2003a). The precise molecular mechanism of this cross-talk between the two pathways is still unknown. It may involve Siah-2, a component of the β -Catenin destruction complex, whose transcription is activated by Wnt-5 (Topol et al., 2003). Another possible candidate is the transcription factor NF-AT, which when phosphorylated by Wnt/ Ca^{2+} signaling in *Xenopus* embryos, enters the nucleus and antagonizes Wnt/ β -Catenin signaling (Saneyoshi et al., 2002). A third regulator of Wnt/ β -Catenin signaling is the MAP (mitogen-activated kinase protein) kinase (MAPK) signaling pathway. In the genetic analysis of endoderm formation in *C. elegans*, *lit-1* and *mom-4* have been demonstrated to activate Wnt/ β -Catenin signaling (Meneghini et al., 1999). *Lit-1* encodes a homologue of the MAPK-related Nemo-like Kinase (Nlk) and *mom-4* a homologue of Tak-1 (TGF- β -activated kinase). Biochemical analysis shows that Tak-1 phosphorylates Nlk, which in turn phosphorylates the Tcf/Lef transcription factors causing the disruption of the DNA-binding ability of the Tcf/Lef- β -Catenin complex (Ishitani et al., 1999; Meneghini et al., 1999).

Btg/Tob proteins define a family of evolutionarily conserved proteins, named after its founding members. Btg-1 (B-cell translocation gene 1) was discovered as a chromosomal translocation in a case of B-cell chronic lymphocytic leukemia (Rouault et al., 1992) and Tob (Transducer of ErbB-2) as an interaction partner of the tyrosine kinase receptor ErbB-2 (Matsuda et al., 1996). By now, six family members, Tob, Tob2 and Btg1, Btg2/TIS21/PC3, Btg3/ANA, and Btg4/PC3B have been identified in higher vertebrates (Matsuda et al., 2001; Tirone, 2001), but their function remains unknown. All Btg/Tob proteins are characterized by the presence of an N-terminal “antiproliferative” domain. Exogenous expression of Btg/Tob proteins in NIH3T3 cells suppresses cell growth by inhibiting G1 progression of the cell cycle via down-regulation of *cyclin D1* transcription (Yoshida et al., 2003a). It has been proposed that Btg/Tob proteins may be regulators of gene transcription. They associate with transcription factors, such as Caf1 (Rouault et al., 1996; Ikematsu et al., 1999; Prevot et al., 2001; Yoshida et al., 2001), HoxB9 (Prevot et al., 2000), and, as in the case of Tob, with members of the Smad transcription complex

(Yoshida et al., 2000; Tzachanis et al., 2001; Yoshida et al., 2003b). Tob is the only family member whose function has been studied by loss-of-function in mouse using homologous recombination. Mice lacking functional Tob protein develop an osteopetrotic phenotype and are predisposed to cancer (Yoshida et al., 2000; Tzachanis et al., 2001; Yoshida et al., 2003a).

The starting point for the work reported here was a large-scale cDNA macroarray screen of *Xenopus* aimed at isolating Spemann organizer genes (Wessely et al., 2004). Multiple isolates of *chordin*, *paraxial protocadherin* (*PAPC*), and a novel gene were among the genes up-regulated by both maternal β -Catenin and *Xenopus* Nodal-related (*Xnr*). This novel gene, designated *xBtg-x*, is presented in this paper. It is a new member of the Btg/Tob gene family, most closely related to Btg-3 and Btg-4. *xBtg-x* is expressed in the Spemann organizer, and its overexpression induces complete axis duplication and a total rescue of the effects of UV ventralization in the embryo.

Material and methods

Embryo manipulations and whole-mount in situ hybridization

Xenopus embryos obtained by in vitro fertilization were maintained in 0.1 \times modified Barth medium (Sive et al., 2000) and staged according to Nieuwkoop and Faber (1994). UV irradiation and LiCl treatment were performed as described (Fainsod et al., 1994). Synthetic mRNA injections were performed at the 4- or 8-cell stage. For synthetic mRNA synthesis, pCS2-*xBtg-x*, pCS2-*cer-S* (Agius et al., 2000), pCS2- *β -Catenin-myc* (Yost et al., 1996), and pCS2-*Nlk* (Ishitani et al., 1999) were linearized with *NotI* and transcribed with Sp6 RNA polymerase (Piccolo et al., 1999). pCDNA- *ΔN -XTcf-3* (Molenaar et al., 1996) was linearized with *XbaI* and transcribed with T7 RNA polymerase. The sequences of the antisense morpholino oligomers used in this study were 5'-GGG ACA AAG ATG CTC ATT TTA ACA G-3' (*xBtg-MO1*) and 5'-GCC ACT ATC TCT TCA ATC ATC TCC G-3' (*xBtg-MO2*). Antisense morpholino oligomers were diluted to a concentration of 1 mM. A total of 8 nl of a mixture of *xBtg-MO1*, *xBtg-MO2*, and water at a ratio of 1:1:2 was used for *Xenopus* injections at the 2- to 4-cell stage.

Ectodermal explants were excised at blastula stage 9 and cultured in 0.5 \times MMR saline (Sive et al., 2000) until sibling embryos reached the required stage. Embryos and explants were processed for RT-PCR analysis as described (Sasai et al., 1995). The following primer pair was used for *xBtg-x*: 194 bp, forward 5'-CAC CGG GAT TGG AAC AGT-3', reverse 5'-GTG GCC GGT GTA TCT CCT-3'. Additional RT-PCR primer sets, PCR conditions used, and the protocol for the whole-mount in situ hybridizations can be found at <http://www.hhmi.ucla.edu/derobertis>.

Protein analysis

In vitro transcription/translation was performed using the TNT® SP6 Coupled Reticulocyte Lysate System (Promega) and PRO-MIX® L-[³⁵S] in vitro cell labeling mix (Amersham). The β -Catenin stabilization study was performed in Western blots stained with the monoclonal anti-myc antibody 9E10 (Roche) diluted to 1:1000. Proteins were extracted as previously described (Liu et al., 1999).

Luciferase assays in *Xenopus* and human 293T kidney cells

For reporter assays, *Xenopus* embryos were injected at the 4-cell stage four times into the animal pole with a mixture of 20 pg luciferase reporter construct and 10 pg pRL-CMV (Renilla luciferase, Promega) as internal standard for transfection efficiency. At the 8-cell stage, a subset of these embryos was re-injected with 3.2 ng *xBtg-x* mRNA. Ectodermal explants were excised at stage 9 and cultured in $0.5 \times$ MMR until sibling embryos reached stage 10. Five explants were lysed in 50 μ l Passive Lysis Buffer (Promega) and cleared by centrifugation. Luciferase activity was measured from 20 μ l extract using the Dual-Luciferase® Reporter Assay System (Promega) and the LB 96V MicroLumat Plus (EG&G Berthold Technologies).

For reporter gene experiments in tissue culture, 293T cells were seeded into 24-well plates. A total of 1 μ g plasmid DNA was combined with 33 ng luciferase reporter construct (TOP-flash, FOP-flash) and 33 ng pRL-CMV as internal control and transfected with 2 μ l FuGENE 6 (Roche). Cells were harvested after 48 h, lysed in 100 μ l Passive Lysis Buffer (Promega), and 20 μ l was used to determine luciferase activity. For all experiments, luciferase activity was presented as fold induction calculated using the ratio of firefly to Renilla luciferase. Transfections were performed in triplicate and experiments repeated at least two times.

Results

Isolation and expression of *xBtg-x*

We recently described a comprehensive genome-wide screen analyzing gene expression in *Xenopus* by differential hybridization of cDNA macroarrays (Wessely et al., 2004). Probes were generated from gastrula stage embryos in which two major embryonic regulatory pathways, Nodal-related and maternal β -Catenin signaling, were activated or inhibited. Differential probes were then hybridized to nylon filters containing more than 72,000 cDNAs from a gastrula stage library. Changes in gene expression levels for each individual clone were then visualized in a single graph showing β -Catenin dependence on the *x* axis and Nodal-related dependence on the *y* axis (Wessely et al., 2004). As shown in Fig. 1A, in this “combined organizer graph,” genes regulated by both

β -Catenin and Nodal-related signaling such as *chordin* are located in the upper right-hand quadrant, while genes regulated only by β -Catenin signaling such as *Xnr-3* are found to the right along the *x* axis. To identify novel Spemann organizer genes, clones localized in the upper right-hand quadrant were sequenced. Interestingly, multiple cDNAs encoded a previously undescribed member of the Btg/Tob gene family, *xBtg-x* (indicated in red in Fig. 1A, GenBank Accession Number AY762636). Btg/Tob proteins are characterized by an antiproliferative domain consisting of two conserved sub-domains, “Box A” and “Box B” (Fig. 1B). In *Xenopus*, three Btg/Tob family members were known: two alleles of *Xenopus* Btg-4 (*xBtg-4*) that were originally described as the maternal B9.10 and B9.15 p30 proteins (GeneBank Accession Number X73317 and X73316), *xBtg-1* (Saka et al., 2000), and *xTob-2* (Yoshida et al., 2003b). Phylogenetic comparison of *xBtg-x* showed that the clone was novel, yet related to Btg-3 and -4 (Fig. 1C). Since not all members of the Btg/Tob gene family have been identified in *Xenopus*, the new clone was named *xBtg-x*.

In the course of the macroarray screen, nine independent clones of *xBtg-x* were identified by sequencing cDNAs located in the upper right quadrant of the “combined organizer graph” (red spots in Fig. 1A). cDNAs in this region were expected to be expressed in the Spemann organizer at gastrula (Wessely et al., 2004), and whole-mount in situ hybridization of gastrula stage embryos showed that *xBtg-x* was indeed expressed in the dorsal mesendoderm (Figs. 1E, J). We next confirmed that the maternal β -Catenin and Nodal-related signaling pathways regulate expression of *xBtg-x* in organizer tissue. Treatment of embryos with LiCl at the 32-cell stage, which induces β -Catenin stabilization (Klein and Melton, 1996), caused radial expression of *xBtg-x* in the marginal zone (Fig. 1F). Embryos microinjected with synthetic mRNA encoding a dominant-repressive version of the transcription factor *XTcf-3* (ΔN -*XTcf-3*), which no longer binds to β -Catenin and therefore constitutively inhibits maternal β -Catenin signaling (Molenaar et al., 1996), inhibited *xBtg-x* expression in dorsal mesendoderm (Fig. 1G). We showed that Nodal-related signaling is also required for the dorsal expression of *xBtg-x* at gastrula using embryos microinjected at the 4-cell stage with *cer-S* mRNA (Fig. 1H). Cer-S is a specific antagonist of mesoderm-inducing Nodal-related molecules in *Xenopus* and interferes with mesoderm induction and Spemann organizer formation (Agius et al., 2000; Wessely et al., 2001).

Developmental RT-PCR analysis of 4-cell stage to early tailbud stage embryos demonstrated that *xBtg-x* was a zygotically expressed gene first detected after mid-blastula transition expressed at high levels through blastula and gastrula stages and then declining to very low levels (Fig. 1D). Whole-mount in situ hybridization analysis showed that at blastula stage (stage 9) high levels of *xBtg-x* mRNA were found throughout the

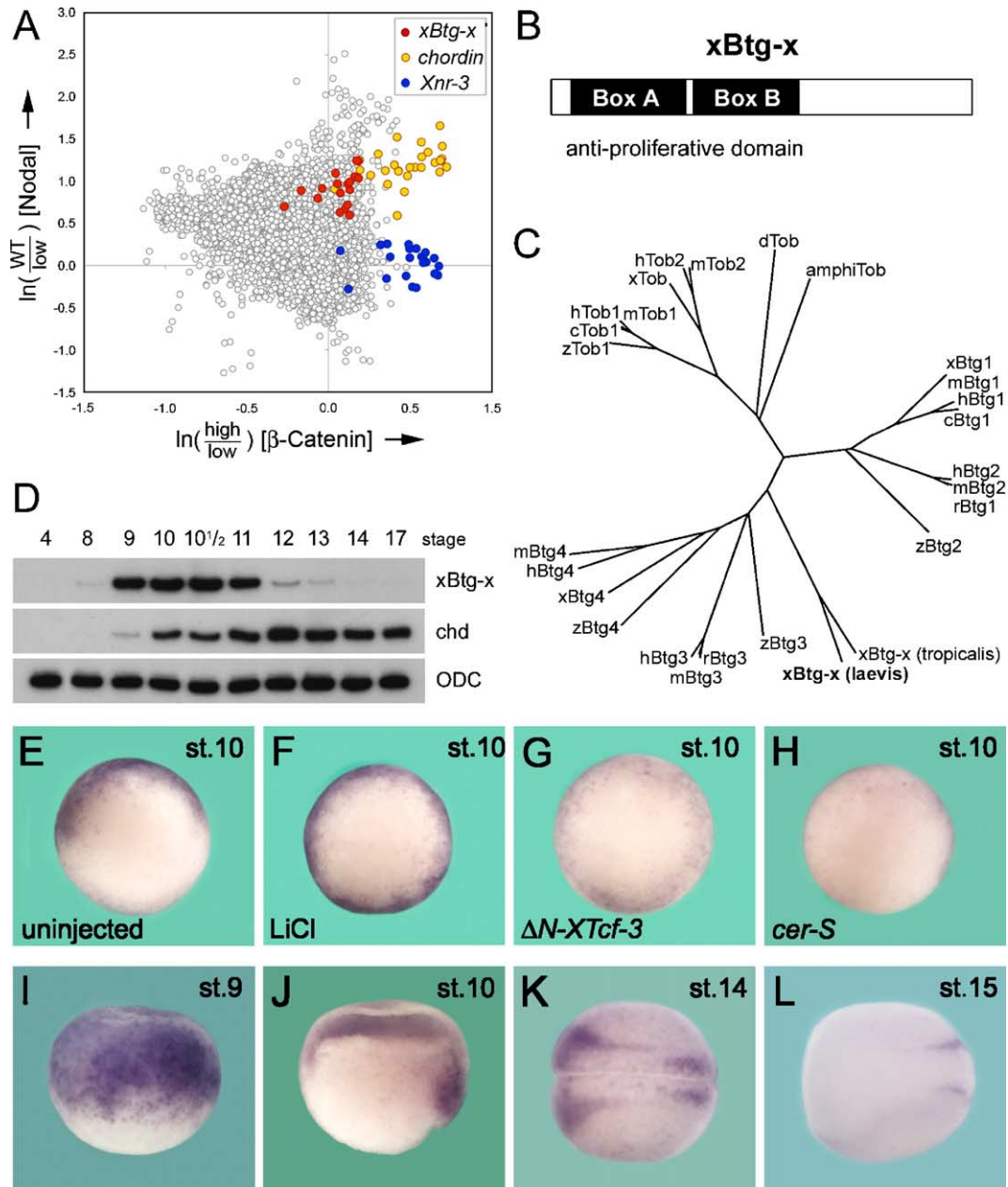


Fig. 1. Cloning and expression analysis of *xBtg-x*. (A) The “combined organizer graph” of clones regulated by maternal β -Catenin (x axis) and Nodal-related signaling (y axis) in a cDNA macroarray screen (Wessely et al., 2004). Each spot represents one cDNA clone analyzed. The positions of *xBtg-x*, *Xnr-3* (a target of β -Catenin), and *chordin* (a target of both β -Catenin and Nodal-related signaling at gastrula) are indicated. (B) Schematic diagram of *xBtg-x* showing the conserved Boxes A and B of the antiproliferative domain characteristic of members of the Btg/Tob gene family. (C) Phylogenetic tree of the Btg/Tob gene family. Alignment was performed using the ClustalW program. (D) RT-PCR analysis of *xBtg-x* and *chordin* expression in stage 4 to stage 17 *Xenopus* embryos. *Ornithine Decarboxylase* (*ODC*) serves as loading control. (E–L) Whole-mount in situ hybridization of *Xenopus* embryos with *xBtg-x* antisense probe. (E–H) Expression of *xBtg-x* in gastrula stage embryos viewed from the vegetal side with dorsal to the top: (E) uninjected control; (F) embryo treated with LiCl at the 32–cell stage; (G) embryo injected with 800 pg ΔN -*XTcf-3* mRNA, or (H) 150 pg of the Nodal-related antagonist *cer-S* mRNA. (I) Expression of *xBtg-x* at stage 9, lateral view; (J) stage 10 hemi-sectioned along the dorso-ventral axis, dorsal to the right; (K) stage 14 and (L) stage 15 in dorsal view, anterior to the left.

animal region (Fig. 1I). Animal cap expression declined at gastrula stage, when *xBtg-x* mRNA was detected in the dorsal mesendoderm (Fig. 1J). At neural plate stage, *xBtg-x* was found in the region adjacent to neural plate in the anterior and the posterior end of the embryo and then became undetectable (Figs. 1K, L).

In summary, we have isolated a new member of the Btg/Tob gene family that displays a developmentally regulated expression pattern during *Xenopus* embryogenesis. At gastrula, *xBtg-x* is expressed in the Spemann organizer, and this expression requires both maternal β -Catenin and Nodal-related signals.

xBtg-x promotes dorsal development

To test whether *xBtg-x* can mimic the function of Spemann's organizer, synthetic *xBtg-x* mRNA was injected into a ventral blastomere at the 4- and 8-cell stage. Interestingly, *xBtg-x* induced ectopic axis formation (55%, $n = 147$) many with complete head structures (Fig. 2B). Histological analysis showed that the ectopic axes contained duplicated notochords, somites, and an expanded neural tube (Figs. 2D, E). The induction of complete secondary axes suggested that *xBtg-x* may act on the maternal β -Catenin signaling pathway (Wodarz and Nusse, 1998). To

test this hypothesis, we co-injected *xBtg-x* with ΔN -*XTcf-3* mRNA (Molenaar et al., 1996) into a single ventral blastomere and found that ΔN -*XTcf-3* completely blocked the axis inducing activity of *xBtg-x* (Fig. 2C).

In *Xenopus* embryos, maternal β -Catenin signaling can be inhibited by UV irradiation of the recently fertilized egg (Scharf and Gerhart, 1980; Heasman, 1997; De Robertis et al., 2000). UV-treated embryos develop into ventralized embryos devoid of dorsal structures (also called "belly-pieces", Fig. 2F). Strikingly, microinjection of *xBtg-x* mRNA into UV-treated embryos completely rescued dorsal development in 100% cases (Fig. 2G). These results

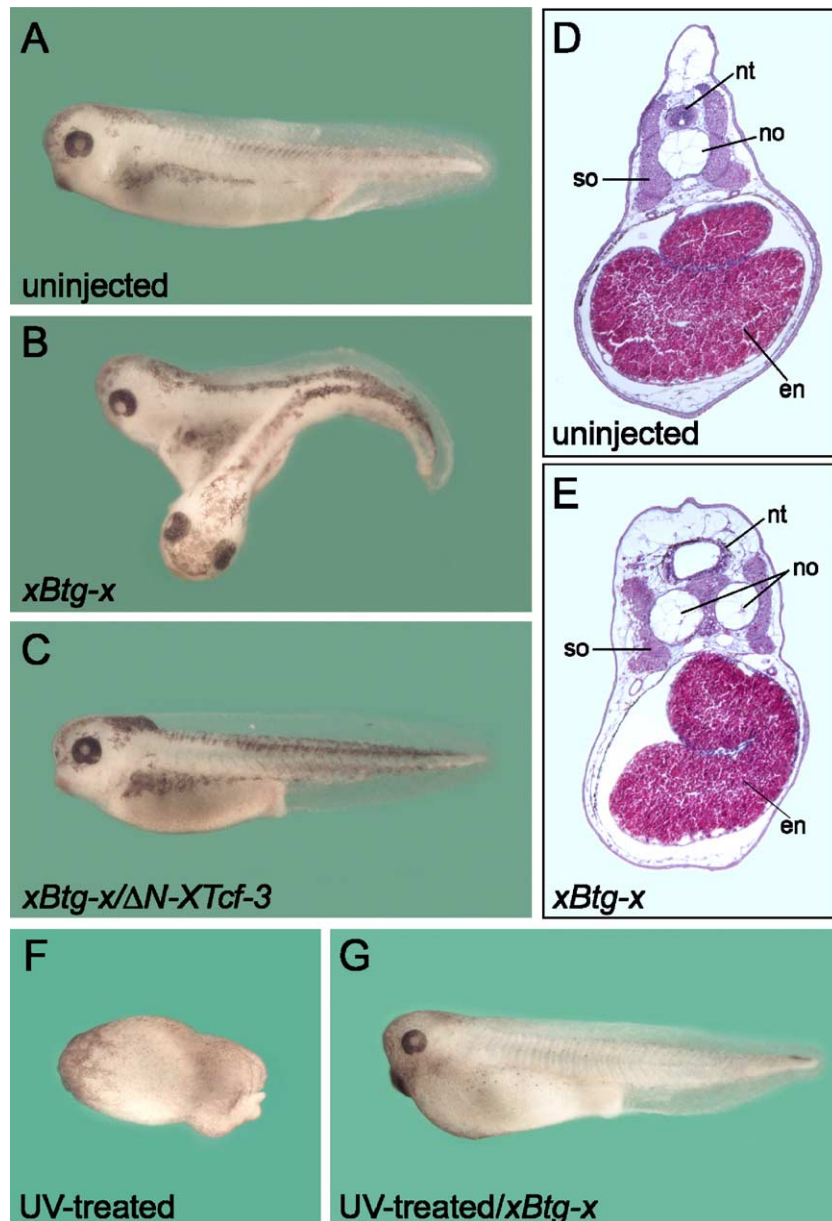


Fig. 2. *xBtg-x* induces complete secondary axis formation and rescues UV ventralization. (A) Uninjected control embryos at stage 40. (B) Axis duplication in an embryo injected at the 8-cell stage once ventrally with 0.8 ng *xBtg-x* mRNA (55%, $n = 147$). (C) Embryo co-injected with *xBtg-x* mRNA and 200 pg ΔN -*XTcf-3* mRNA. Note the absence of ectopic axes (0%, $n = 36$). (D, E) Histological analysis of uninjected and *xBtg-x*-injected embryos; en, endoderm; no, notochord; nt, neural tube; so, somite. (F) Ventralized embryo after UV irradiation. (G) Complete rescue of the ventralized phenotype by a single injection of 0.8 ng *xBtg-x* mRNA at the 8-cell stage.

suggested that *xBtg-x* can activate the maternal β -Catenin pathway in the early frog embryo.

xBtg-x induces the BCNE center

In *Xenopus*, maternal β -Catenin signaling causes the expression of the BMP antagonists *chordin*, *noggin*, and *Xnr-3*, as well as the transcription factor *siamois* in the dorsal animal cap of the blastula stage. This region is known as the Blastula Chordin and Noggin Expressing (BCNE) center and is dependent on β -Catenin signaling (Wessely et al., 2001; De Robertis and Kuroda, 2004; Kuroda et al., 2004). *xBtg-x* mRNA was microinjected into the animal region at the 4-cell stage. At blastula stage, uninjected embryos expressed *Xnr-3* and *chordin* transcripts confined to the dorsal-animal (BCNE) region (Figs. 3A, C). *xBtg-x* mRNA injected embryos greatly expanded *Xnr-3* expression all over the animal cap (Fig. 3B). Similarly, *chordin* was up-regulated, although its expression was less uniform than *Xnr-3* (Fig. 3D). This difference may be explained by the fact that *Xnr-3* is a direct target gene of maternal β -Catenin signaling, while the expression of *chordin* is likely regulated via the β -Catenin target gene *siamois* (McKendry et al., 1997; Wessely et al., 2004).

The induction of BCNE center genes by *xBtg-x* was confirmed by RT-PCR of ectodermal explants (Fig. 3E). *Xenopus* embryos were injected four times animally at the 4-cell stage with *xBtg-x*, ΔN -*XTcf-3*, or a combination of both mRNAs. As is the case for other genes that activate Wnt/ β -Catenin signaling (Wessely et al., 2001), *xBtg-x* induced the expression of the known BCNE genes, *chordin*, *noggin*, *siamois*, and *Xnr-3*, but not the dorsal mesodermal genes *goosecoid* (*gsc*) and *Xenopus Nodal-related 1* (*Xnr-1*) (Fig. 3E, lane 4). Co-injection of ΔN -*XTcf-3* mRNA completely blocked this response, indicating that this

activity of *xBtg-x* mRNA depends on the Wnt/ β -Catenin pathway (Fig. 3E, lane 5). Interestingly, when the *xBtg-x*-injected ectodermal explants were cultured until tailbud stage and then analyzed by RT-PCR, up-regulation of the neural markers *NCAM* and *Rx2a* was detected (Fig. 3F).

Activation of Wnt-dependent gene transcription by *xBtg-x*

We next used luciferase reporter gene assays to confirm that *xBtg-x* indeed acts on the Wnt/ β -Catenin pathway. The pXNR3-Luc reporter containing 294 base pairs upstream of the *Xnr-3* initiation codon is sufficient to mimic the Wnt-response of the *Xnr-3* promoter (McKendry et al., 1997). pXNR3-Luc was injected together with a Renilla luciferase expression construct (pRL-CMV) as an internal standard into the animal pole of 8-cell stage embryos. In the absence of *xBtg-x*, animal cap explants contained only low luciferase activity. However, upon co-injection of *xBtg-x* mRNA relative luciferase activity increased 4-fold (Fig. 4A). These results were confirmed with the TOP-flash luciferase Wnt/ β -Catenin reporter containing three copies of the TCF binding site upstream of a thymidine kinase (TK) minimal promoter and a luciferase open reading frame (Korinek et al., 1997). TOP-flash luciferase was also activated by *xBtg-x* in microinjected animal caps (Fig. 4B). This activation was dependent on Wnt/ β -Catenin signaling via Tcf/Lef-1, since *xBtg-x* did not activate expression of the FOP-flash luciferase construct, in which the Tcf/Lef-1 binding sites are mutated (Korinek et al., 1997).

xBtg-x also elicited a Wnt/ β -Catenin signaling response in an established mammalian system. Human 293T kidney cells were transiently transfected with the Top-flash luciferase reporter (Fig. 4C). In the absence of stimulus, 293T cells show low luciferase activity. In contrast to *Xenopus* animal caps, transient transfection of *xBtg-x* alone

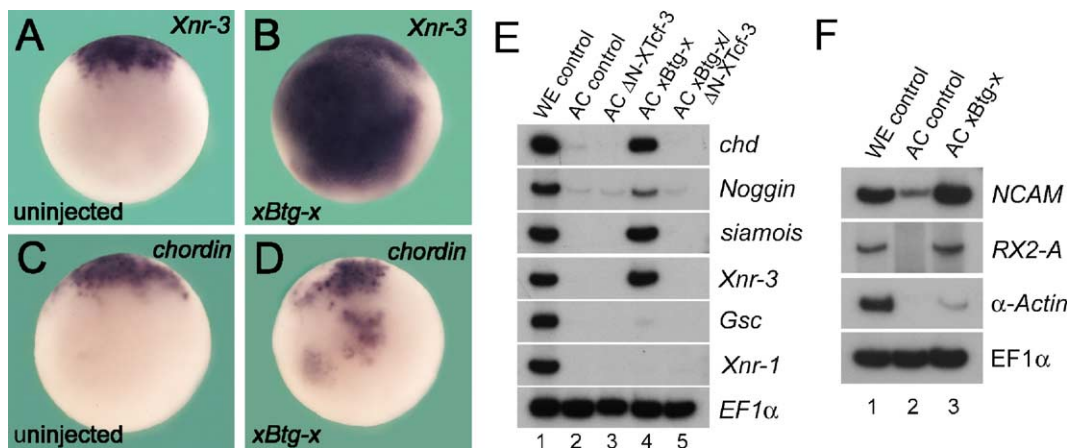


Fig. 3. *xBtg-x* activates the BCNE center gene expression in *Xenopus* embryos. (A–D) Whole-mount in situ hybridization of blastula stage 9 embryos with *Xnr-3* (A, B) and *chordin* probes (C, D). Embryos were injected four times animally with 0.8 ng *xBtg-x* mRNA (B, D). Note the *xBtg-x*-induced expansion of *Xnr-3* and *chordin* expression throughout the animal cap at blastula. All embryos are shown in animal view with dorsal to the top. (E) RT-PCR analysis of uninjected whole embryos (WE) and animal cap (AC) explants at stage 10 of *Xenopus* embryos left untreated (lane 2), injected into the animal region with 800 pg ΔN -*XTcf-3* (lane 3), 3.2 ng *xBtg-x* (lane 4), or ΔN -*XTcf-3* and *xBtg-x* mRNA (lane 5). *EF-1 α* is a control for equal loading. (F) RT-PCR analysis of ectodermal explants at stage 20. Note that *xBtg-x* mRNA functions as a neural inducer.

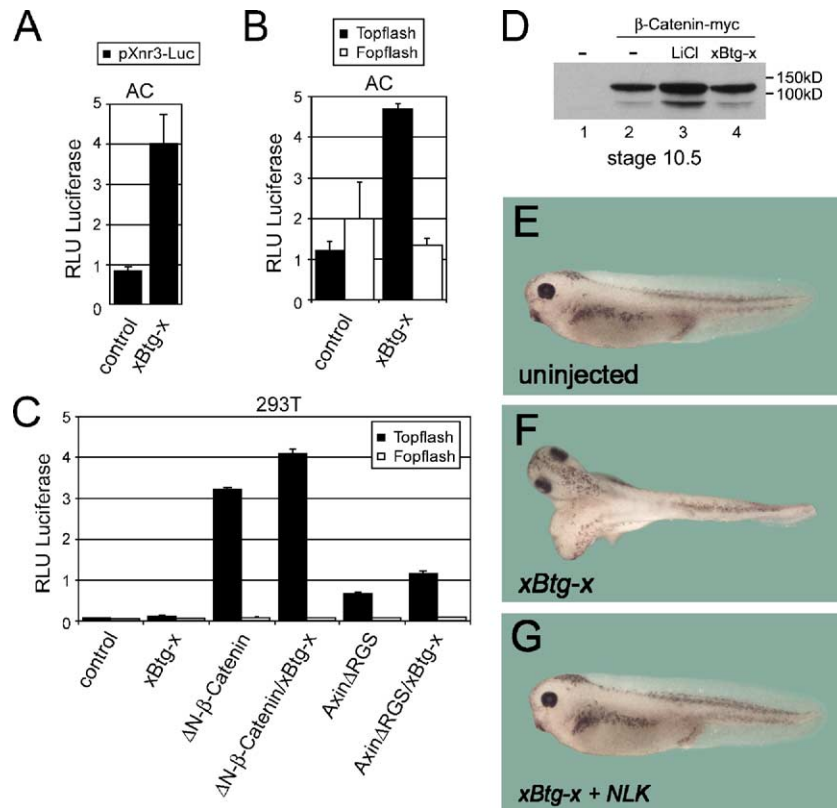


Fig. 4. *xBtg-x* activates the Wnt/ β -Catenin signaling pathway. (A) Luciferase assays of gastrula stage animal cap explants from *Xenopus* embryos injected with a *Xnr-3* promoter luciferase construct. (B) Animal caps microinjected with the TOP-flash or FOP-flash Wnt/ β -Catenin reporter alone or co-injected with 3.2 ng *xBtg-x* mRNA. (C) Luciferase reporter assay using the TOP-flash or FOP-flash Wnt/ β -Catenin reporter in human 293T kidney cells transfected with *xBtg-x*, stabilized β -Catenin (ΔN - β -Catenin), *xBtg-x* and ΔN - β -Catenin, dominant-negative Axin (Axin Δ RGS), or Axin Δ RGS together with *xBtg-x*. (D) Western blot analysis of gastrula stage *Xenopus* embryos injected into the animal pole with 150 pg β -Catenin-myc mRNA at the 4-cell stage. Subsets of the injected embryos were then either left untreated, treated with LiCl at the 32-cell stage, or injected with 3.2 ng *xBtg-x* mRNA at the 8-cell stage. Note that *xBtg-x* mRNA does not significantly stabilize β -Catenin protein. (E) Uninjected control *Xenopus* embryo at stage 40, (F) embryo microinjected once ventrally at the 8-cell stage with 0.8 ng *xBtg-x* mRNA showing a partial secondary axis (50%, $n = 24$) and (G) injected with a combination of *xBtg-x* and 60 pg *Nlk* mRNA. Note that double axis formation by *xBtg-x* is blocked by co-injection of *Nlk* mRNA (0%, $n = 18$).

did not significantly increase TOP-flash luciferase activity in 293T cells (Fig. 4C and data not shown). However, upon co-transfection of a stabilized form of β -Catenin, ΔN - β -Catenin (Baker et al., 1999), *xBtg-x* moderately increased the effect of ΔN - β -Catenin (Fig. 4C). A similar increase of the TOP-flash luciferase reporter in response to *xBtg-x* was observed when the Wnt/ β -Catenin pathway was activated by dominant-negative Axin (Axin Δ RGS) (Zeng et al., 1997) or dominant-negative GSK-3 β (Pierce and Kimelman, 1995) (Fig. 4C and data not shown) rather than by β -Catenin itself. All these effects were dependent of Tcf/Lef1, since no activation was seen in the case of the FOP-flash luciferase reporter (Fig. 4C).

The data from human 293T cells indicated that *xBtg-x* cannot activate the Wnt/ β -Catenin signaling on its own, but rather requires the presence of stabilized β -Catenin to activate the TOP-flash reporter gene. Thus, we next tested whether *xBtg-x* mRNA can increase the levels of β -Catenin itself. *Xenopus* embryos injected with β -Catenin-myc mRNA were co-injected with *xBtg-x* mRNA, treated at the 32-cell stage with LiCl or left untreated, and β -Catenin levels analyzed at blastula stage using an anti-myc antibody

(Fig. 4D). As expected, inhibition of endogenous GSK-3 β activity by LiCl resulted in elevated levels of β -Catenin protein (Fig. 4D, lane 3). However, injection of *xBtg-x* mRNA did not change steady-state levels of β -Catenin-myc protein (Fig. 4D, compare lanes 2 and 4). Taken together, the reporter gene assays support the hypothesis that axis duplication by *xBtg-x* mRNA results from the activation of canonical Wnt/ β -Catenin in *Xenopus* embryos. However, *xBtg-x* does not appear to increase β -Catenin protein levels.

Nlk is a serine/threonine protein kinase that negatively controls β -Catenin-dependent transcription by regulating the DNA-binding activity of the β -Catenin/TCF complex in the nucleus (Ishitani et al., 1999; Meneghini et al., 1999; Ishitani et al., 2003). Since *xBtg-x* is proposed to function downstream of β -Catenin stabilization in the Wnt/ β -Catenin signaling pathway (Fig. 4D), we asked whether Nlk could counteract the effect of *xBtg-x* on Wnt signaling. To test this hypothesis in *Xenopus*, embryos were injected at the 8-cell stage once ventrally either with *xBtg-x* or with a combination of *xBtg-x* and *Nlk* mRNA and allowed to develop until tailbud stage. Embryos injected with *xBtg-x* alone developed secondary axes, but no ectopic structures could be

observed when *Nlk* mRNA was co-injected (Figs. 4E–G). Similarly, *Nlk* mRNA inhibited the induction of the β -Catenin target genes, *chordin*, *Xnr-3*, and *siamois* in ectodermal explants, microinjected with *xBtg-x* mRNA (data not shown).

Taken together, these data suggest that *xBtg-x* regulates Wnt/ β -Catenin signaling downstream of the stabilization of β -Catenin at the level of the Tcf/Lef- β -Catenin complex.

xBtg-x regulates neural plate convergent-extension movements

Next, we analyzed the function of *xBtg-x* during *Xenopus* development in a loss-of-function approach using antisense morpholino oligomers. Since *Xenopus laevis* is allotetraploid, two pseudo-alleles of *xBtg-x* exist. All nine isolates from the macroarray screen corresponded to one allele, and the sequence of the second allele was identified in the public EST databases (UniGene Cluster XI.24168). The nucleotide sequences of both alleles around the translational start site were too divergent to be targeted by a single antisense morpholino oligomer, and therefore two oligomers (*xBtg-MO1*, *xBtg-MO2*) were designed. As shown in Fig. 5A, in vitro translation of the approximately 30 kDa *xBtg-x* protein was efficiently inhibited by *xBtg-MO1*. When either *xBtg-MO1*, *xBtg-x-MO2*, or a mixture of the two antisense

morpholino oligomers (*xBtg-MO1* + 2) were injected into 2-cell stage *Xenopus* embryos, the embryos displayed a significant delay in the closure of the neural tube (data not shown). Since the phenotype of the individual antisense morpholino oligomers was weaker than the mixture of both, we used the mixture of *xBtg-MO1* and *xBtg-x-MO2* in subsequent experiments (Figs. 5B–K). Histological analyses of *xBtg-MO1* + 2-injected embryos at stage 40 showed that neural tube, notochord, and somites were still differentiated after the initial delay in neural tube closure (Figs. 5B, C). This suggested that convergent-extension movements of neural plate cells towards the midline might be affected. Molecular marker analysis supported this hypothesis. *HoxB9*, a posterior neural marker was widened (Figs. 5D, E), and *Sox-2*, a marker of the entire neural plate, was expanded laterally in *xBtg-MO1* + 2-injected embryos (Figs. 5F, G). Similarly, expression of *pax-3* in the lateral neural plate (Bang et al., 1997), as well as expression of *slug* in neural crest cells (Mayor et al., 1995), was shifted away from the midline at the neural plate stage (Figs. 5H–K). It is noteworthy that, despite the delay in neural tube closure, the timing of the expression of *pax-3* and *slug* and other regional specific neural markers, such as the forebrain marker *otx-2*, the hindbrain marker *krox-20*, and the spinal cord marker *hoxB9*, was not affected (Figs. 5D–K). The phenotype of the *xBtg-MO1* + 2 in the neural

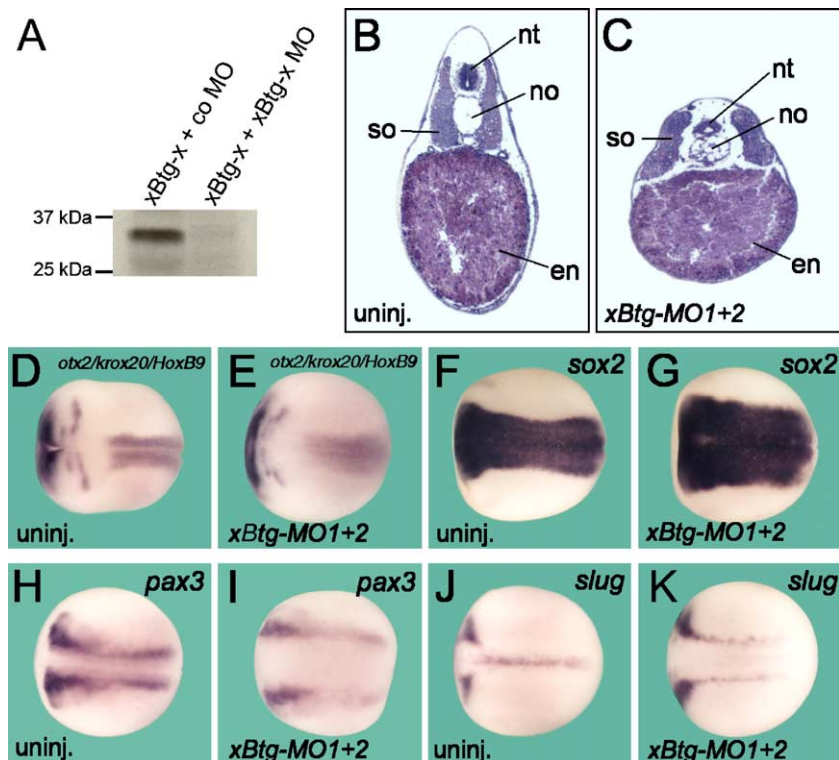


Fig. 5. Phenotype of *xBtg-x* antisense morpholino oligomer injections. (A) In vitro transcription/translation of pCS2-*xBtg-x* in the presence of control morpholino and *xBtg-x-MO1*. (B) Histological analysis of uninjected control embryos and (C) embryos injected at the 2-cell stage with *xBtg-x-MO1* + 2 at stage 40; en, endoderm; nt, neural tube; no, notochord; so, somites. (D–K) Whole-mount in situ hybridization of uninjected and *xBtg-x-MO1* + 2 injected embryos at neurula stage 14. (D, E) Combination of *Otx2*, *Krox20*, and *HoxB9* probes; (F, G) *Sox-2*; (H, I) *Pax3*; (J, K) *Slug*. Note that *xBtg-x-MO1* + 2 inhibits neural plate convergence and extension movements.

plate correlated well with the expression of *xBtg-x* in the anterior and posterior edges of the neural plate (Figs. 1K, L). When the *xBtg-MO1+2* injected embryos were analyzed at blastula stage, no changes in the expression of *chordin* or *Xnr-3* in the BCNE center could be observed (data not shown).

In summary, while the antisense morpholino oligomer experiments did not reveal an early developmental role for *xBtg-x*, they indicated that *xBtg-x* function is required for neural tube convergence and closure.

Discussion

Btg/Tob proteins were originally described as antiproliferative proteins inhibiting cell cycle progression (Matsuda et al., 2001; Tirone, 2001). In *Xenopus*, two family members have been previously reported. *xBtg-1* is a target of the T-Box transcription factor *Xbra* and regulates gastrulation movements (Saka et al., 2000). *xTob-2* binds to SMAD molecules and is a modulator of BMP activity (Yoshida et al., 2003b). In this study, we present a new member of the Btg/Tob gene family, *xBtg-x*, which was isolated in the course of a large-scale screen for genes expressed in the Spemann organizer.

In the case of *xBtg-x*, microinjections of its mRNA into *Xenopus* embryos resulted in phenotypes consistent with a role in Wnt/ β -Catenin signaling: (1) *xBtg-x* mRNA induced the formation of complete secondary axes, and this phenotype could be inhibited by co-injection of ΔN -*XTcf-3*, a deletion mutant of *XTcf-3* that cannot interact with β -Catenin and thereby constitutively represses Wnt/ β -Catenin-dependent transcription (Figs. 2B, C). (2) In UV-irradiated embryos, in which dorsal development is lost due to inhibition of the maternal β -Catenin signaling pathway, *xBtg-x* mRNA injection completely rescued dorsal axial development (Fig. 2G). (3) Microinjection of *xBtg-x* mRNA into the animal region at the 4-cell stage induced ectopic expression of the β -Catenin-responsive genes *Xnr-3* and *chordin*, and this induction was dependent on β -Catenin, since it was inhibited by the co-injection of the dominant-repressive ΔN -*XTcf-3* (Fig. 3E). (4) *xBtg-x* activated the Wnt/ β -Catenin-responsive TOP-flash luciferase reporter in *Xenopus* animal caps and human 293T kidney cells, but did not activate the control construct, in which the Tcf/Lef binding sites had been mutated (Figs. 4B, C).

xBtg-x and Wnt/ β -Catenin signaling

The precise function of *xBtg-x* within the Wnt/ β -Catenin signaling pathway remains unknown. Microinjection of *xBtg-x* mRNA did not result in apparent changes in β -Catenin protein levels suggesting that *xBtg-x* acts downstream of proteins such as Axin and Gsk-3 β that are involved in stabilizing β -Catenin. However, we have been unable to demonstrate direct biochemical interactions

between *xBtg-x* and components of the Wnt/ β -Catenin signaling pathway such as β -Catenin, Axin, or XTcf-3 (data not shown).

An indication for a mechanism of action for *xBtg-x* comes from the comparison of the *Xenopus* and tissue culture results. Microinjection of *xBtg-x* mRNA into early *Xenopus* embryos activated the Wnt/ β -Catenin signaling pathway, while in human 293T cells, transfection of *xBtg-x* only activated the Wnt/ β -Catenin-dependent TOP-flash luciferase reporter in the presence of stabilized β -Catenin activity (Fig. 4C). It is possible that the *Xenopus* *xBtg-x* protein does not retain full activity when transfected into human cells, and therefore the transfection experiments in human 293T cells will have to be repeated once the human homologue of *xBtg-x* has been identified. However, a second more appealing hypothesis is that the status of Wnt/ β -Catenin signaling is context-dependent and that in the early frog embryo another signaling pathway prevents the stabilized β -Catenin from being transcriptionally active. Microinjection of *xBtg-x* mRNA might be able to release this antagonism. One possible candidate pathway is non-canonical Wnt/ Ca^{2+} signaling. In zebrafish, knock-down experiments of non-canonical Wnt-5 result in dorsalized embryos due to ectopic activation of canonical Wnt/ β -Catenin signaling throughout the embryo (Westfall et al., 2003a). Maternal-zygotic Wnt-5 mutant zebrafish embryos also displayed elevated levels of stabilized β -Catenin (Westfall et al., 2003a). However, embryos microinjected with *xBtg-x* mRNA did not stabilize β -Catenin (Fig. 3J) making it unlikely that *xBtg-x* signals through the non-canonical Wnt/ Ca^{2+} signaling pathway in *Xenopus*. Another possible target of *xBtg-x* activity is the MAP Kinase Nlk, which regulates the stability of the Tcf/Lef- β -Catenin transcription complex by phosphorylating Tcf/Lef bound to DNA (Ishitani et al., 1999). We show here that the axis-inducing activity of *xBtg-x* is blocked by the co-injection of *Nlk* mRNA (Fig. 4G). In *C. elegans*, Nlk constitutes a very important regulatory component of the canonical Wnt/ β -Catenin pathway (Meneghini et al., 1999). However, loss-of-function experiments in *Xenopus* and zebrafish using antisense morpholino oligomers do not support a role for Nlk in early axis specification, but have rather suggested a role for Nlk in the formation and patterning of the mesoderm (Ohkawara et al., 2004; Thorpe and Moon, 2004). Clearly, more research will be needed to unravel the detailed molecular mechanism by which *xBtg-x* activates the early β -Catenin signaling pathway.

In vivo role of *xBtg-x*

Microinjection of antisense morpholino oligomers targeting the two *xBtg-x* pseudo-alleles delayed neural plate closure as evidenced by the expression pattern of *sox2*, *pax-3*, and *slug* (Figs. 5F–K). The onset of this phenotype correlated with the expression domain of *xBtg-x* at the border of the neural plate (Figs. 1K, L). It has been shown

that several distinct morphogenetic movements contribute to neural tube closure (Colas and Schoenwolf, 2001; Zohn et al., 2003). The planar cell polarity (PCP) signaling pathway regulates the convergent-extension movements of the midline (Park and Moon, 2002; Wallingford and Harland, 2002; Sasai et al., 2004). Molecules such as the actin-binding protein Shroom are required within the neuroepithelium for neural plate bending by regulating the apical constriction of the neuroepithelium (Haigo et al., 2003), and canonical Wnt/ β -Catenin signaling is required at the border of the neural plate (Saint-Jeannet et al., 1997). Interfering with these processes in *Xenopus* embryos results in a phenotype similar to the one observed with the xBtg-x antisense morpholino oligomers (Park and Moon, 2002; Wallingford and Harland, 2002; Sasai et al., 2004).

In contrast to this role in neural plate formation, a function for xBtg-x in the maternal β -Catenin signaling pathway initiating dorso-ventral patterning of the early *Xenopus* embryo was not revealed by our knock-down experiments. Injection of xBtg-x antisense morpholino oligomers did not ventralize *Xenopus* embryos nor did it alter the expression domains of β -Catenin target genes such as *Xnr-3* or *chordin*. It is therefore possible that xBtg-x might not by itself be involved in early embryonic patterning. Instead, the over-expression experiments could simply mimic the activity of other family members. However, the robust and tightly regulated early expression of xBtg-x mRNA in the animal cap and the dorsal mesendoderm suggest an early function for xBtg-x. One explanation for the absence of an early phenotype is that at least three other members of the Btg/Tob gene family are expressed during this time of development. *xTob2* (Yoshida et al., 2003b), *xBtg-1* (Saka et al., 2000), and *xBtg-3* (our unpublished observations) could compensate for the absence of xBtg-x protein. Interestingly, injections of an antisense morpholino oligomer against *xTob2* also did not result in any developmental abnormalities in *Xenopus* embryos (Yoshida et al., 2003b). Therefore, a final judgment for the importance of xBtg-x at the blastula/gastrula stage will have to await simultaneous elimination of multiple family members.

Concluding remarks

The current study identified xBtg-x in the course of a comprehensive genome-wide screen for cDNAs expressed in the Spemann organizer. While the Btg/Tob gene family has been known for more than 10 years, the molecular mechanism by which the individual proteins function is still unknown. Using microinjection into *Xenopus* embryos, we could show that xBtg-x mRNA is a strong activator of the Wnt/ β -Catenin signaling pathway regulating its activity at the level of the Tcf/Lef- β -Catenin transcriptional complex. This study exemplifies how the manipulations possible in the *Xenopus* embryo help to identify novel biological activities of known and unknown proteins or protein families. Such test systems are especially important in the

genomic era, where genome-wide analyses identify a multitude of genes with largely unknown biological activity.

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