Author correction


The authors apologise for an error that occurred in Fig. 1A and its legend. In Fig. 1A, the chimeric β-Engrailed construct should not have been depicted or described as metabolically stabilized (β-catenin’s N-terminal domain did not contain stabilizing point mutations). This does not alter the results presented, the discussion or the conclusions. If a stabilized form of β-Engrailed were generated and compared with the non-stabilized form employed in this study, it would be expected to display a greater potency in blocking canonical Wnt signals at equivalent injected doses.

The error appeared in both the print and the pdf versions of this article. The correct Fig. 1 is shown below.
A β-catenin/engrailed chimera selectively suppresses Wnt signaling

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SUMMARY

β-catenin plays an integral role in cell-cell adhesion by linking the cadherin complex of the adherens junction to the underlying actin cytoskeleton. In addition, β-catenin transduces intracellular signals within the Wnt developmental pathway that are crucial to the proper establishment of embryonic axes and pattern formation of early mesoderm and ectoderm. For example, in the context of a defined dorsal ‘organizer’ region of early Xenopus embryos, β-catenin enters the cortical actin cytoskeleton in association with additional proteins such as siamois, a homeobox gene contributing to the specification of the dorsoanterior axis, are activated. To further examine the role that β-catenin plays in Wnt signaling, we generated a chimeric protein, β-Engrailed (β-Eng), in which the C-terminal trans-activation domain of β-catenin is replaced with the transcriptional repression domain of Drosophila Engrailed. Dorsal overexpression of this mRNA in early Xenopus embryos leads to suppression of organizer-specific molecular markers such as siamois, Xnr-3 and goosecoid, corresponding with the dramatic morphological ventralization of embryos. Ventralized embryos further exhibit reduced activity of the Wnt pathway, as indicated by the loss of the notochord/organizer marker, chordin. Importantly, β-Eng associates and functions normally with the known components of the cadherin complex, providing the experimental opportunity to repress β-catenin’s signaling function apart from its role in cadherin-mediated cell-cell adhesion.

Key words: Xenopus, β-catenin, Cadherin, Engrailed, Wnt, signalling, Cell-cell adhesion

INTRODUCTION

β-catenin, the vertebrate orthologue of Drosophila Armadillo (McCrea et al., 1991; Peifer et al., 1991), is a versatile molecule that functions in multiple cellular capacities (Barker et al., 2000; Hatzfeld, 1999) including cadherin-dependent cell-cell adhesion (Barth et al., 1997; Peifer, 1995; Steinberg and McNutt, 1999). Within adherens junctions, β-catenin links cadherin to the cortical actin cytoskeleton in association with additional proteins such as α-catenin (Herrenknecht et al., 1991; Nagafuchi and Tsukita, 1994) and α-actinin (Knudsen et al., 1995; Ozawa, 1998). In common with integrins and other adhesion systems, cadherin function is believed to be modulated by such cytoskeletal interactions (Alattia et al., 1999). In addition to its role at the plasma membrane, β-catenin is an essential participant in the Wnt signal transduction pathway (Dierick and Bejsovec, 1999; Gumbiner, 1995), wherein it physically associates with certain members of the High Mobility Group (HMG) of transcription factors, namely Lef/Tcf (Barker et al., 2000; Behrens et al., 1996), to activate gene transcription (Cavallo et al., 1997; Clevers and van de Wetering, 1997). In its role as a signaling molecule, β-catenin is critical in effecting axis specification and pattern formation in the early vertebrate embryo (Funayama et al., 1995; Miller and Moon, 1996; Molenaar et al., 1996; Sokol, 1999). β-catenin is characterized by a central Armadillo domain that is composed of 12 imperfect Arm repeats, each of approximately 42 amino acids, which facilitate interactions with various protein partners (Huber et al., 1997; Peifer et al., 1994). For example, at the plasma membrane, β-catenin’s Armadillo domain binds a highly conserved region within cadherin’s cytoplasmic tail. In the nucleus, β-catenin’s Armadillo domain associates with the N-terminal region of Lef/Tcf transcription factors (Behrens et al., 1996; Cavallo et al., 1997; van de Wetering et al., 1997). β-catenin’s Armadillo domain is flanked on either side by dissimilar N- and C-terminal domains, which confer additional functionality (Barth et al., 1996; Munemitsu et al., 1996). For example, the N-terminal domain participates in regulating β-catenin signaling through its Wnt-dependent phosphorylation by GSK-3 on specific serine residues (Yost et al., 1996), resulting in β-catenin’s destabilization and reduced signaling capacity (Dominguez et al., 1995; Fagotto et al., 1997; Pierce and Kimelman, 1995).

During early Xenopus development, Wnt signaling functions to increase the cellular level of β-catenin in dorsal blastomeres (Fagotto et al., 1997; Gradi et al., 1999; Schneider et al., 1996; Wylie et al., 1996), establishing an organizing center through the direct activation of specific genes such as the homeobox gene siamois (Bramon et al., 1997; Fan et al., 1998; Lemaire and Kodjabachian, 1996) and Xnr-3 (McKendry et al., 1997; Smith et al., 1995). Indirectly, β-catenin/Wnt signals also modulate expression of the dorsal and organizer specific genes goosecoid (Cho et al., 1991; Laurent et al., 1997; Steinbeisser et al., 1993) and chordin (Sasai et al., 1994). Collectively, these
orchestrated transcriptional events aid in patterning the dorsalventral and anteroposterior axis of the developing embryo. The developmental power of the Wnt pathway has been illustrated by misexpression of each of the known components of the signaling pathway. For example, the overexpression of β-catenin in ventral blastomeres induces dorsal mesoderm and generates an ectopic organizer capable of specifying an ectopic dorsal axis (Funayama et al., 1995; Guger and Gumbiner, 1995; McCrea et al., 1993). Furthermore, expression of β-catenin is capable of rescuing the dorsal axis following antisense depletion of maternal β-catenin or ultra-violet radiation (Funayama et al., 1995; Heasman et al., 1994), or the imposition of upstream blockades of the Wnt signal transduction cascade, demonstrating β-catenin’s key role in the Wnt signaling pathway and axis specification (Deardorff et al., 1998; Itoh et al., 1998; Sokol, 1996).

In the absence of Wnt signals, the stability of the signaling pool of β-catenin is limited by a large protein complex including APC, Axin/Conductin, GSK-3, and components of the ubiquitination/proteasome pathway (Bienz, 1999; Easwaran et al., 1999; Ikeda et al., 1998; Kikuchi, 1999; Kishida et al., 1998; Rubinfeld et al., 1996; Salomon et al., 1997). Wnt signals prohibit the activity of GSK-3 within this complex, resulting in β-catenin’s reduced ubiquitin/proteasome-mediated degradation, and thus its increased stability and signaling capacity (Aberle et al., 1997).

The Wnt family is comprised of secreted glycoprotein ligands that bind members of the frizzled family of transmembrane receptors (Dale, 1998; Moon et al., 1997; Shulman et al., 1998). Frizzled receptor activation leads to GSK-3 inactivation in a manner that is incompletely understood, but which requires the Dishevelled protein, itself a complex entity capable of activating parallel and distinct signaling pathways (Boutros and Mlodzik, 1999; Sokol, 1996). β-catenin-dependent Wnt signaling ultimately requires β-catenin’s entry into the nucleus and direct association with architectural transcription factors of the HMG box family (Clevers and van de Wetering, 1997; Nusse, 1997). Furthermore, β-catenin’s C-terminal transactivation domain (Vleminkx et al., 1999) facilitates expression of Wnt target genes including siamois and Xnr-3.

Since β-catenin acts in more than one capacity within the cell, primarily as a mediator of cadherin-dependent cell-cell adhesion and signaling via the Wnt pathway, it has been experimentally challenging to distinguish between β-catenin’s functional contributions in either cellular or developmental contexts (Cox et al., 1996; Fagotto et al., 1996; Gumbiner, 1995; Zhu and Watt, 1999). Lacking, thus far, has been an experimental reagent capable of selectively blocking signaling mediated via β-catenin, as opposed to other upstream or downstream components of the Wnt pathway. To address this problem, we have generated a chimeric protein in which the C-terminal transactivation domain of β-catenin has been replaced with the active repression domain of Drosophila Engrailed (Jaynes and O’Farrell, 1991; Smith and Jaynes, 1996), while leaving β-catenin’s N-terminal and Armadillo domains intact. Similar approaches employing Engrailed-repressor fusions have been successfully used to block fushi tarazu (Smith and Jaynes, 1996a), Xbra (Conlon et al., 1996) and siamois (Fan and Sokol, 1997; Kessler, 1997) mediated gene transcription. The β-catenin/Engrailed construct contains three S→A and one T→A, N-terminal mutations (Fig. 1), which prevent its phosphorylation via GSK-3 and subsequent ubiquitin/proteasome-mediated degradation (Yost et al., 1996). While leaving cadherin-mediated adhesion unperturbed, we find that this chimera, termed β-Engrailed (or β-Eng), successfully suppresses β-catenin’s specific function within the Wnt signaling pathway, indicated by the graphic ventralization of Xenopus embryos as assessed by both molecular and morphological criteria. We expect that β-Engrailed will prove useful in multiple studies in which the specific repression of β-catenin mediated Wnt signaling is required.

**MATERIALS AND METHODS**

**Xenopus embryo manipulations**

Xenopus females were induced to produce eggs by injection of 800 units of human chorionic gonadotropin at least 10 hours prior to their isolation. Eggs were obtained by standard means, placed in 0.1× MMR (Marc’s modified Ringers solution, pH 7.4: 100 mM NaCl, 2 mM KCl, 2 mM CaCl2, 1 mM MgCl2, 5 mM Hepes), and fertilized in vitro. The jelly coat was removed by incubation of the embryos prior to initial cleavage by brief room temperature incubation in a solution of 2% cysteine HCl (pH 8.0). Blastula cleavage stages and dorsal versus ventral polarity were determined according to the method of Nieuwkoop and Faber (1967). Borosilicate glass microinjection pipettes (capillary tubes; Sutter Instrument Co., Novato, CA, USA) were pulled using a P-30 pulling instrument (Sutter), bevelled with a K. T. Brown Type Micropipette beveller (Sutter), and embryos microinjected using the NA-1 oil-driven microinjector (Sutter). Microinjection of RNA was performed at 2-, 4- and 8-cell cleavage stages into either one or two blastomeres in the subequatorial region of the respective marginal zones unless otherwise stated, and embryos placed within a solution of 5% Ficoll in 1× MMR for at least 60 minutes prior to transfer to 0.1× MMR for culture at 14-22°C. mRNAs were injected in volumes ranging from 10-40 nl per blastomere, and in concentrations ranging from 50 pg-3 ng. Embryonic phenotypes were evaluated using a standard binocular dissecting microscope (Nikon, model SMZ-U). Anteroposterior and dorsoventral embryonic axis development was evaluated using the DAI index (Kao and Elionson, 1988).

**RNA and in vitro transcription**

The β-Eng (β-Engrailed) repressor chimera was constructed using standard and PCR-based subcloning techniques (Fig. 1A). A construct of Drosophila Engrailed was kindly provided by Heithem El-Hodiri (Jaynes and O’Farrell, 1991). A cDNA construct, β-cateninMT, containing β-catenin’s N-terminal and Armadillo domains and excluding its C terminus, was obtained from pSP36T+MT (bp’s 1-2085) (Funayama et al., 1995) using PCR-based subcloning (forward: 5'-AAG GAA AAA AGC GGC CGC G GCA ACT CAA GCA GAT-3', reverse: 5'-ATT CCC G GGC CCG TGC AGC AGC CTC ATT CTA CCA-3'). An N-terminal NotI site and a C-terminal Apal site were introduced, and the construct inserted into pCDNA3.1+MT using corresponding NotI and Apal sites, placing the construct immediately downstream and in-frame with a myc epitope tag. Next, the N-terminal repressor domain of Engrailed (amino acids 4-300) was isolated via PCR, employing primers that incorporated NotI sites, placing the construct immediately downstream of the C-terminal truncated β-catenin. To facilitate the in vitro transcription of RNA, the entire myc-tagged β-Eng construct was

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moved into pCS2+ (Rupp et al., 1994) at a StuI site using blunt-end ligation, creating β-engMT. The construct’s reading frame was verified by cDNA sequencing at the institutional core sequencing facility.

A non-epitope-tagged construct, β-eng, was produced using PCR, permitting the introduction of the restriction site, CclI (5' end) and EcoRI (3' end) restriction sites (forward: 5'-CCATGGATGACTACAA GCA GAT-3'; reverse: 5'-CGGAATTC TTA CTT GCA CAG GGA TTC-3'). A control construct consisting of only the Engrailed repressor, engMT, was generated using PCR (forward: 5'-GGGGCC AGAT GC TGCTGAG ACG CCA-3'; reverse: 5'GGGGCCC TTA CTT GCA CAG GGA TTC-3'), introducing Apal sites to facilitate insertion into the StuI site of pCS2+MT expression vector. In addition, an untagged construct of the Engrailed repressor domain, engR, was generated via PCR amplification of the repressor region, introducing an 5' BamHI site (forward: 5'-CG GGA TAC ATG GCC CTG GAG CAT C-3') and a 3' EcoRI site (reverse: 5'-G GA TTC TTA CTT GCA CAG GGA TTC-3') for the corresponding insertion into pCS2+. Additionally, XTcf-3/HA, an epitope-tagged version of XTcf-3 (a kind gift of the Clevers laboratory, University of Utrecht, The Netherlands) was generated via N-terminal fusion with three HA repeats. The XTcf-3 cDNA was PCR subcloned from the original plasmid, pCDNAXTcf-3, introducing 5' and 3' XbaI sites using the primers, forward: 5'-GC TCT AGA ATG CCA CTA AAC AGC-3'; reverse: 5'-GC TCT AGA ATG CCA CTA AAC AGC-3'; this fragment was then inserted into pCS2+3HA downstream of three HA repeats at a convenient XbaI site in the first polylinker. Capped mRNAs encoding the aforementioned constructs were generated using the SP6 mMessage mMachine Kit (Ambion, Austin, TX, USA). The cDNA templates were linearized for transcription using the following enzymes: β-engMT (NsiI), β-eng (NsiI), engR (NsiI), engMT (NsiI), pCS2+MT (NcoI), β-cateninMT (NcoI) and XTcf-3/HA (NcoI). The quantity and quality of transcribed RNA products were evaluated on the basis of migration within 1% agarose formaldehyde gels and by optical density (OD 280/260).

Western blots and immunoprecipitation

To evaluate exogenous protein expression following RNA microinjection and in vivo translation, embryos were injected at the 1- and 2-cell stage with myc- and/or HA-tagged constructs. Embryos were cultured until stage 9-10, lysed at 4°C in 15 mM Tris, pH 6.8, and extracted with Freon (1,1,2-trichlorotrifluoroethane) to remove yolk proteins. The lysis buffer was supplemented with a protease inhibitor cocktail consisting of 1 mM phenylmethylsulfonyl fluoride, 4 μg/ml aprotinin, 1 μg/ml peptatin A, 2 μg/ml leupeptin,10 μg/ml antipain, 50 μg/ml benzamid, 10 μg/ml soybean trypsin inhibitor, 100 μg/ml iodoacetamide and 40 μg/ml TLCK. Immunoprecipitations of the myc-tagged constructs, C-cadherin and α-catenin was performed with stage 10-12 embryos extracted in 0.5% Triton X-100, 10 mM Hepes, pH 7.4, 150 mM NaCl, 2 mM EDTA and 0.02% NaN₃ in addition to the aforementioned protease inhibitors. The extract was cleared by centrifugation for 30 minutes, at 4°C, at 14,000 g. Immunoprecipitations of the myc-tagged constructs were performed with a monoclonal anti-myc antibody 9E10, obtained from the Developmental Studies Hybridoma Bank maintained by the University of Iowa. C-cadherin was immunoprecipitated with a polyclonal antibody generated against the extracellular domain of Xenopus C-cadherin (kind gift of W. Brieger and B. Gumbiner, Memorial Sloan-Kettering Cancer Center; Yap et al., 1997). α-Catenin was immunoprecipitated with a monoclonal antibody raised against amino acids 729-906 of mouse α-catenin (Transduction Laboratories, Franklin Lakes, NJ, USA). Immunoprecipitation of the tagged XTcf-3 construct was performed with the monoclonal anti-HA antibody, 12CA5 (Wadzinska et al., 1992). In some experiments, Concanavalin A (Sigma, St Louis, MO, USA) conjugated to sepharose beads, was used as a control for immunoprecipitation of membrane glycoproteins such as cadherin.

Proteins were resolved on 8% SDS-PAGE and transferred to 0.45 μm nitrocellulose membranes. Whole cell lysates were loaded at 2
embryo equivalents per lane, while immunoprecipitates were loaded with at least 2.5 embryo equivalents per lane. To detect proteins in western blots, the following antibodies were used at the following dilutions: 9E10 anti-myc antibody at a 1:10,000 dilution; 12CA5 antibody at 1:5,000; anti-α-catenin antibody at 1:2,500; anti-C-cadherin at 1:2,500. β-Eng constructs and endogenous β-catenin were further detected with a polyclonal anti-β-catenin antibody raised against the N-terminal domain of Xenopus β-catenin (McCrea et al., 1993). Protein bands were visualized via enhanced chemiluminescence (ECL: Amersham, Buckinghamshire, UK) by reacting with either goat anti-rabbit IgG or goat anti-mouse IgG secondary antibodies (1:3000), each conjugated to horseradish peroxidase (Bio-Rad, Hercules, CA, USA).

Cell adhesion assays

Cell adhesion assays were performed according to Torres et al. (1996), with the following minor modifications. Animal caps were excised from stage 9 embryos and dissociated in 1x CaCl\textsuperscript{2}/MgCl\textsuperscript{2}-free modified Barth’s solution (CMF-MBS: 88 mM NaCl, 1 mM KCl, 2.4 mM NaHCO\textsubscript{3}, 10 mM Hepes, pH 7.5) for 1 hour in 35 mm Petri dishes coated with 1% agarose in the aforementioned buffer. Cells were reaggregated at room temperature on an orbital bench-top shaker at 60 rpm for 1 hour in 2 mM Ca\textsuperscript{2+} in the CMF-MBS buffer. Cell reaggregation was observed by digital capture of random cell populations from each sample. Aggregates were scored using the particle analysis utility of NIH Image 1.60 software (1998) (NIH, Bethesda, MD, USA) to scan the digitally captured images for small (5-8 cells) and large (>8 cells) aggregates (Torres et al., 1995).

RT-PCR analysis

Embryos were injected with a total of 0.5 ng of β-engMT mRNA, or β-galactosidase (or left un.injected), at the 4-cell stage in either two dorsal or two ventral blastomeres, or in all four blastomeres. Total RNA was obtained by extracting whole embryos cultured to stage 10-12 with Trizol (Gibco-BRL, Gaithersburg, MD, USA) according to the manufacturer’s protocol, followed by the preparation of mRNA for the reverse transcriptase reaction (Gibco-BRL). The total cDNA levels per sample were normalized prior to PCR Supermix (Gibco-BRL). The total cDNA levels per sample were normalized prior to PCR Supermix (Gibco-BRL) amplification. Controls for DNA contamination were evaluated using PCR in the absence of reverse transcriptase (RT–). Molecular marker primer sequences employed are as follows: Siamois (Fagotto et al., 1997); Histone H4 (Niehrs et al., 1994); Histone H4 (Niehrs et al., 1994); Chordin (Sasai et al., 1997); Xbra (Smith et al., 1991); Xnr-3 (Darras et al., 1997); ornithine decarboxylase (Basséez et al., 1990).

Whole-mount in situ hybridization

Whole-mount in situ hybridizations were undertaken for the molecular marker goosecoid according to Harland (1991), with minor modifications according to El-Hodiri et al. (1997). Embryos were evaluated at the gastrula stage.

Luciferase assays

Luciferase assays were performed according to the method of Brannon et al. (1997); reporter plasmids were the kind gift of M. Brannon and David Kimelman, University of Washington). The experiments were repeated at least three times, and each assay was performed in triplicate. Two reporter plasmids were used: SO1324 and S0, which each included the proximal –0.8 kb promoter of the siamois gene. SO1324 contains three Lef/Tcf binding sites (0, 1 and 3) while the S0 reporter, a negative control, is mutant at these sites and thus prohibited activation by the β-catenin/Tcf transcriptional machinery (Brannon et al., 1997).

Histology and immunohistochemistry

Embryos were cultured to the required stage for each experiment and fixed in MEMPFA (4% paraformaldehyde, 4 mM Mops, 2 mM EGTA, 1 mM MgSO\textsubscript{4}, pH 7.4) for 1-2 hours at room temperature. Fixed embryos underwent serial dehydration and were embedded in paraffin according to published procedures (Kelly, 1991), and sectioned on a rotary microtome (Reichert HistoStat, model 820) at a thickness of 8 μm. Immunohistochemistry was performed using an alkaline phosphatase anti-alkaline phosphatase (APAAP) complex method, according to the manufacturer’s instructions (DAKO APAAP Kit-System 40 K670, DAKO Inc., Carpenteria, CA, USA). Histological sections were evaluated using a standard binocular microscope (Zeiss Axioskop).

RESULTS

β-Eng is effectively expressed and associates normally with the Cadherin complex

To verify expression of β-Eng in early Xenopus embryos, we employed a polyclonal antibody raised against the N terminus of β-catenin (McCrea et al., 1993). A myc-epitope tagged version of β-Eng (β-EngMT) was evaluated by western analysis of injected embryo extracts, revealing that β-EngMT was effectively expressed in a dose-dependent manner (Fig. 1B). The untagged version, β-Eng, was found to be similarly expressed in the early embryo.

To assess if β-Eng associated normally with components of the cadherin complex (cadherin<>β-catenin<>α-catenin), we conducted immunoprecipitation analysis. 2-cell stage embryos were injected with a total of 1.0 ng of β-EngMT mRNA into both blastomeres and then assayed for the association of β-EngMT protein with endogenous C-cadherin. As anticipated, β-EngMT coprecipitated with endogenous C-cadherin (Fig. 1C) and vice versa. Con A (Sigma), a lectin that binds membrane glycoproteins such as cadherin, likewise efficiently coimmunoprecipitated β-EngMT (data not shown). Coimmunoprecipitation experiments indicating that β-EngMT coprecipitated α-catenin and vice versa, confirmed that β-EngMT associated properly with its known binding partners within the cadherin complex (Fig. 1C). Finally, it was demonstrated that β-EngMT held no selective advantage or disadvantage over endogenous β-catenin for incorporation into the cadherin complex, as indicated in the coimmunoprecipitation of C-cadherin and α-catenin. Embryos injected with β-EngMT compared to uninjected embryos contained equivalent amounts of cadherin complexes measured as coimmunoprecipitates of C-cadherin in which α-catenin is detected via western analysis (Fig. 1C, α-catenin blot: lanes 5 and 6) and vice versa (Fig. 1C, C-cadherin blot: lanes 3 and 4).

Expression of β-Eng prevents dorsal development in Xenopus embryos

To examine whether β-Eng could inhibit Wnt signaling required for the formation of the dorsal axis, the chimera was expressed in the organizer region of early Xenopus embryos.
Embryos were injected with 0.5 ng of β-eng or β-engMT mRNA in the subequatorial marginal zone of each dorsal blastomere at the 4-cell stage. In either case, the embryos developed normally through gastrulation, and importantly, no change in cell-cell adhesion was evident (Fig. 2A,B). Though blastopore lip formation was not as well defined as in control (β-gal or uninjected) embryos, closure of the blastopore did occur. Immediately following blastopore closure, however, injected embryos failed to extend along their anteroposterior axis, remaining spherical in shape and devoid of neural pigmentation and anterior structures (Fig. 2C versus D). The ventralized phenotype was observed in 78% of the dorsally injected β-engMT embryos (n=326), while controls were normal, displaying an average Dorso-Anterior Index (DAI) score of 5 (n=500; Table 1). The majority of these β-eng injected embryos lacked formation of a visible neural plate and, in the most severe cases, remained completely spherical even at the tailbud control stages (55% of ventralized embryos, DAI=0-1; Tables 1, 2) (Fig. 2E versus F). Many β-Eng expressing embryos displayed the formation of large cysts, similar to those observed in embryos ventralized via the

Table 1. Phenotype data

<table>
<thead>
<tr>
<th>mRNA injected</th>
<th>Normal</th>
<th>Ventralized</th>
<th>Other defect</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-eng</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dorsal</td>
<td>22</td>
<td>78</td>
<td>0</td>
<td>326</td>
</tr>
<tr>
<td>Ventral</td>
<td>58</td>
<td>0</td>
<td>42</td>
<td>209</td>
</tr>
<tr>
<td>engMT</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Dorsal</td>
<td>89</td>
<td>0</td>
<td>11</td>
<td>37</td>
</tr>
<tr>
<td>Ventral</td>
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<td>0</td>
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<td>39</td>
</tr>
<tr>
<td>Control</td>
<td>95</td>
<td>0</td>
<td>5</td>
<td>500</td>
</tr>
</tbody>
</table>

Xenopus embryos were injected with 0.5 ng of mRNA in both dorsal or ventral blastomeres at the 4-cell stage. Embryos were scored at the tailbud stage for the development of the dorsal axis and dorsal structures as either Normal, indistinguishable from the control-equivalent stage, Ventralized or Other defect, which included gastrulation defects, etc. Control embryos were either β-galactosidase injected or un.injected.

Table 2. DAI scores for β-Eng ventralized embryos

<table>
<thead>
<tr>
<th>DAI score</th>
<th>% of ventralized embryos</th>
<th>% of control embryos</th>
</tr>
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<tbody>
<tr>
<td>0</td>
<td>34</td>
<td>–</td>
</tr>
<tr>
<td>1</td>
<td>21</td>
<td>–</td>
</tr>
<tr>
<td>2</td>
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<td>3</td>
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<tr>
<td>4</td>
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<td>–</td>
</tr>
<tr>
<td>5</td>
<td>0</td>
<td>100</td>
</tr>
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</table>

Average DAI score 1.4 5.0

β-eng injected embryos scored as Ventralized (DAI<5) in Table 1, were further evaluated for the degree of ventralization according to the dorso-anterior index (Kao and Elinson, 1988).

n=254 ventralized embryos.
antisense depletion of β-catenin mRNA (Heasman et al., 1994). Less severely affected embryos were nonetheless ventralized, with obvious perturbations of anterior and dorsal structures, including a crude and diminutive dorsal fin (43% of ventralized embryos, DAI=2-3; Table 2). Overall, β-Eng expressing embryos have been classified with a DAI score of 4 or less (average DAI=1.4; Table 2; Kao and Elinson, 1988). We expect that the expression of β-eng mRNA in dorsal blastomeres specifically inhibits Wnt signaling, and thus dorsal axis development in Xenopus embryos. While injection of three control constructs for the Engrailed repression domain yielded some nonspecific gastrulation abnormalities (distinct from those observed in β-Eng expressing embryos), less than 1% produced ventralized embryos (Table 1).

β-Eng does not significantly alter Ca²⁺-dependent cellular adhesion

To rule out the possibility that the observed phenotype might result in part from β-Eng’s perturbation of cadherin-mediated cell-cell adhesion, Ca²⁺-dependent cellular adhesion assays were performed. Embryos were injected after the first cleavage in the animal pole with either β-eng or β-catenin mRNA in both blastomeres. The embryos were cultured to stage 9 and then animal caps expressing β-Eng, β-catenin or uninjected controls were each dissociated in a Ca²⁺-free medium and reaggregated in the presence or absence of Ca²⁺ (Torres et al., 1995). Relative cell-cell adhesion was evaluated by comparing the ability of the cells to form small (5-8 cells) versus large (greater than 8 cells) aggregates in the presence of Ca²⁺ (Kemler et al., 1989). As indicated in Table 3, the ability of cells expressing β-Eng to form either small or large aggregates is not significantly altered in comparison to β-catenin expressing or uninjected cells. The total number of aggregates formed in the Ca²⁺(+) case is similar in each assay (Table 3, total aggregates: β-Eng, 98; β-catenin, 97; control, 103). As for uninjected and β-catenin expressing cells (Table 3, control and β-catenin), the addition of Ca²⁺ significantly increased cell aggregation in β-Eng expressing cells (Aberle et al., 1996; Fagotto and Gumbiner, 1994), demonstrating the restoration of cadherin-mediated cellular adhesion. Even cells injected with a twofold increase over the ventralizing dose of β-eng mRNA (1.0 ng) formed aggregates to a similar extent in the presence of Ca²⁺ (Table 3, β-Eng).

In further support of the hypothesis that β-Eng does not alter cadherin-mediated cell-cell adhesion, it should also be noted that there are no observable defects in β-Eng expressing embryos as they develop and undergo gastrulation, a developmental process known from numerous studies to be sensitive to changes in cell-cell adhesion (Kintner, 1992; Levine et al., 1994). Furthermore, manual dissection and sectioning of embryos expressing β-Eng does not reveal any gross internal perturbations of blastomere adhesion. These observations are in contrast to embryos expressing p120 catenin or various other cadherin constructs, such as a dominant-negative cadherin, which failed gastrulation and had readily observable deficiencies in cell-cell adhesion (Lee and Gumbiner, 1995; Paulson et al., 1999).

Taken together, the adhesion data suggests that β-Eng does not significantly modify cell-cell adhesion by perturbing the cadherin complex, further supporting the hypothesis that β-Eng’s effects are primarily mediated via its suppression of β-catenin-dependent Wnt signaling.

Histological analysis reveals tissue differentiation and loss of dorsal morphology in β-Eng expressing embryos

Histological analysis of dorsally injected β-eng embryos revealed that the three germ layers were present (endo-, meso- and ectoderm; Fig. 2I), while formation of dorsal structures such as neural tube, notochord, and somites was significantly perturbed, and in severe cases, inhibited (Fig. 2I versus J). In control-equivalent tailbud stage embryos (injected dorsally with β-Eng; DAI<4), anterior structures such as eyes, cement gland and head, were significantly reduced, and histological sections revealed severely perturbed neural structures (Fig. 2IK versus J.L) and a greatly reduced dorsal axis. These observations are in congruence with the hypothesis that β-Eng is interfering with organizer-dependent dorsalization, but not specification of the germ layers. Further, histology supports our morphological and molecular work indicating that β-Eng suppresses dorsal axial structures and expression of the corresponding molecular markers.

β-Eng expression results in the reduction of dorsal and organizer specific molecular markers

To test for the inhibition of Wnt signaling at the molecular level, we examined the effect of expressing β-Eng on organizer-specific gene expression (Dawid, 1994). Expression of siamois, a homeobox gene and direct target of Wnt/β-catenin signaling, was evaluated using RT-PCR of stage 10 embryos that had been injected with 0.25 ng of β-eng mRNA at the 4-cell stage in each dorsal blastomere (0.50 ng total). Embryos expressing β-Eng protein showed a marked decrease of siamois when compared to β-galactosidase injected and uninjected controls (Fig. 3A). The organizer specific marker, Xnr-3, a member of the TGFB superfamily and direct target of Wnt/β-catenin signaling, and chordin, another organizer/dorsal-specific marker, were also significantly reduced in β-eng dorsally injected embryos (Fig. 3A). Additionally, expression of β-Eng led to a marked reduction in the pan-mesodermal marker, Xbra, indicating a concomitant loss of mesodermal induction with the inhibition of axis formation (Fig. 3A).

Using whole-mount in situ hybridization methods (Harland, 1991), we further tested if goosecoid, an indirect target of Wnt/β-catenin signaling, was repressed. Goosecoid is an organizer-specific marker normally expressed along the dorsal

<table>
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<th>Table 3. Adhesion assays: cell adhesion analysis</th>
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<tr>
<td>mRNA injected</td>
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<td>----------------</td>
</tr>
<tr>
<td>Number of aggregates</td>
</tr>
<tr>
<td>5-8 cell</td>
</tr>
<tr>
<td>&gt;8 cell</td>
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<tr>
<td>Total aggregates</td>
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</table>

Two-cell embryos were injected with a total of either 1 ng β-Eng mRNA, 2 ng of β-catenin mRNA, or were uninjected (control). All assays were performed in both the presence (+) and absence (−) of calcium. The values indicate the number of aggregates of given sizes counted in three separate samples of each assay. Total aggregates, the number of aggregates counted in three separate samples of each assay.
blastopore lip of gastrulating embryos (Fig. 3B). Embryos at the 4-cell stage injected with 0.5 ng of β-eng into both dorsal or ventral blastomeres were probed with a digoxigenin-labeled goosecoid probe (Harland, 1991). Dorsally injected embryos showed a dramatic decrease in goosecoid expression along the dorsal blastopore lip, reflecting the noted loss of organizer-specific gene expression (Fig. 3B). Ventrally injected embryos, along with un.injected controls, demonstrated no loss of goosecoid expression at the gastrula stage (Fig. 3B). Later in development, goosecoid is normally expressed in anterior/head structures and along the dorsal aspect of the embryo. At control-equivalent tailbud stages, β-eng injected embryos still showed a loss of goosecoid expression, while no loss was apparent in the ventrally injected and control embryos (data not shown). In summary, the reduction of siumois, chordin, goosecoid and Xnr-3 expression in β-eng injected embryos is consistent with the morphological loss of dorsoanterior axial structures (embryonic ventralization), indicating direct effects upon Wnt/β-catenin-dependent signaling and axis induction.

Exogenous β-catenin restores axis development in β-eng injected embryos

β-catenin-mediated rescue of normal axial development in embryos in which Wnt signals have been suppressed provides a powerful specificity test for Wnt pathway inhibitors (Heasman et al., 1994; Kessler, 1997). If β-Eng directly interferes with the normal activity of β-catenin-dependent Wnt signaling within the dorsal organizer, then exogenous β-catenin would be anticipated to restore normal axial development. Indeed, coexpression of 2 ng of β-catenin with 0.5 ng of β-eng results in normal axial development in over 90% (n = 49) of injected embryos (Table 4; Fig. 4A versus B), indicating that β-Eng specifically suppresses β-catenin-mediated Wnt signaling.

To further investigate the mechanism by which this rescue takes place, stage 10 embryos expressing either β-Eng (0.5 ng of mRNA) and XTcf-3 (0.25 ng mRNA), or β-catenin (0.5 ng), XTcf-3 (0.25 ng) and β-catenin (2.0 ng), were assayed via protein communoprecipitation (Fig. 4C, see also Fig. 6B). The molecules were epitope tagged (β-Eng and β-catenin with myc; XTcf-3 with HA) to facilitate the selective biochemical assay of the exogenous proteins and thereby provide an assessment of their relative extent of association. Immunoprecipitation of XTcf-3 indicated that it binds β-Eng (Fig. 4C,1). To a significant extent, coinjection of β-catenin with β-eng revealed β-catenin’s competition for XTcf-3, illustrated by the relative reduction of β-Eng protein communoprecipitating with XTcf-3 (Fig. 4C,2). This evidence suggests that the β-catenin-mediated rescue of β-eng injected embryos occurs via the displacement/competition of β-Eng from XTcf-3, and that β-Eng’s repressive action upon Wnt/β-catenin gene targets is specific.

The specificity of β-Eng in suppression of β-catenin/Wnt signaling is supported by luciferase reporter assays of the known Wnt target, siumois

To further address the specificity and efficacy of β-Eng in inhibiting β-catenin-dependent Wnt signaling, we tested suppression of transcription from siumois reporter constructs. These reporters, generously provided by the laboratory of David Kimelman (University of Washington), consisted of the 0.8 kb proximal promoter of the siumois gene (Brannon et al., 1997). The first construct, S01234, included three Tcf binding sites required for gene activation via β-catenin/Tcf and two general enhancing elements. The second construct, S0, was mutant in all five of these binding sites, and served as a negative control (Fig. 5). The reporters were injected into the vegetal hemisphere of dorsal blastomeres, a region of high endogenous β-catenin signaling. Dorsal injection of the S0 reporter plasmid DNA established the basal level of luciferase transcription, while dorsal injection of S01234 resulted in nearly a 15-fold induction of luciferase activity over basal levels. Coexpression of the S01234 reporter with β-Eng abolished luciferase activity, resulting in an overall induction of only a fraction over basal levels (Fig. 5). Thus, β-Eng effectively suppressed the expression of an established target of β-catenin/Wnt signaling, likely via a direct repressive effect upon its promoter.

Nuclear translocation of β-Eng

Thus far, we have provided evidence that β-Eng specifically suppresses Wnt/β-catenin transcription. To further assess β-Eng’s function, we tested if β-Eng entered the nucleus to bind XTcf-3. Using immunohistochemical techniques, we found that Xenopus embryos injected with β-engMT mRNA stained positively for the inclusion of β-Eng within their nuclei (Fig. 6A, left panel). When compared to control embryos, either uninjected (Fig. 6A, right panel), or with molar equivalents of mRNA encoding only the myc tag (data not shown), nuclear staining was not detected (Fig. 6A, right panel), indicating that the β-Eng protein is capable of translocating to the nucleus to suppress Wnt/β-catenin-mediated transcription. This result was anticipated because β-Eng retains β-catenin’s entire Armadillo domain, which can bind Lef/Tcf transcription factors harboring nuclear localization sequences (NLS) (Huber et al., 1996). Furthermore, it has been shown that β-catenin’s Armadillo repeat domain is the minimal structural requirement for its NLS-independent (Lef/Tcf independent) and importin-independent nuclear localization (Fagotto et al., 1998; Funayama et al., 1995).

β-Eng’s capacity to bind XTcf-3 in vivo is again supported via western analysis, demonstrating a reciprocal association between the two proteins (Fig. 6B). Embryos were coinjected with 500 pg of β-engMT mRNA and 250 pg of XTcf-3/HA in both blastomeres at the 2-cell stage. At stage 9, the embryos were lysed and communoprecipitated using the monoclonal anti-HA 12CA5 antibody and the anti-myc 9E10 antibody. Western analysis of the communoprecipitation revealed a strong and reciprocating association between β-Eng and XTcf-3. This evidence, taken along with the nuclear immunohistochemistry,

<table>
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<th>mRNA injected</th>
<th>Normal</th>
<th>Abnormal</th>
<th>Ventralized</th>
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<tbody>
<tr>
<td>β-eng</td>
<td>18</td>
<td>0</td>
<td>82</td>
<td>–</td>
<td>57</td>
</tr>
<tr>
<td>β-eng/β-catenin</td>
<td>91</td>
<td>9</td>
<td>0</td>
<td>91</td>
<td>45</td>
</tr>
<tr>
<td>Control</td>
<td>87</td>
<td>13</td>
<td>0</td>
<td>–</td>
<td>121</td>
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Xenopus embryos injected with 0.5 ng of mRNA dorsally, yielded ventralized phenotypes (Fig. 4A). Coinjection of 0.5 ng of β-eng mRNA with 2.0 ng of β-catenin mRNA restored the dorsal axis (RA) in over 90% of the injected embryos (Fig. 4B).
implies a direct nuclear role for β-Eng in suppressing β-catenin-dependent Wnt signaling.

**DISCUSSION**

In the normal developing *Xenopus* embryo, maternal β-catenin becomes asymmetrically localized at the dorsal marginal zone of vegetal blastomeres as early as the first cleavage (Larabell et al., 1997) and establishes the region of the embryo that will eventually serve as the organizer. This organizer region patterns the dorsal embryonic axis and specifies various dorsal cell fates (Moon and Kimelman, 1998; Zoltewicz and Gerhart, 1997). In this study, we demonstrate that the expression of a chimeric fusion protein of β-catenin, β-Eng, in which the C-terminal transactivation domain has been replaced with the strong repressor domain of *Drosophila* Engrailed, antagonizes the activity of the organizer, resulting in graphically ventralized embryos lacking a dorsi-ventral axis.

Embryos expressing β-Eng dorsally are morphologically devoid of dorsi-ventral and anteroposterior axial development, as indicated by external gross anatomical features and histological sections. At the molecular level, affected embryos lack significant expression of organizer-specific genes.

It was further demonstrated that the expression of the Engrailed repressor alone does not lead to embryo ventralization phenotypes or association with the XTcf-3 transcription factor (data not shown). It is known that the combined N-terminal and Armadillo domains of β-catenin positively regulate the Wnt pathway, as assessed by dorsal axis induction assays in *Xenopus* embryos (Fagotto et al., 1996; Funayama et al., 1995). β-Eng retains both of these domains (Fig. 1A), yet is a potent suppressor of the Wnt pathway, indicating a specific nuclear function for β-Eng. The specificity...
β-Engrailed selectively suppresses Wnt signaling

of β-Eng was also evident by the suppression of direct β-catenin/Tcf gene targets, siamois and Xnr-3 (Fig. 3), and in the observation that exogenous β-catenin rescued a normal dorsal axis in β-Eng injected embryos. The combination of the morphological observations with the molecular marker data strongly suggests that β-Eng specifically represses β-catenin-mediated Wnt signaling.

While β-Eng suppresses dorsal signals that establish the normal embryonic axes, including the expression of direct targets of β-catenin/Wnt signals (e.g. organizer-specific gene expression), the exact mechanism by which β-Eng acts is not clear. β-catenin is known to accumulate in the nucleus, bind Lef/Tcf transcription factors, and induce the transcription of largely unknown Wnt/β-catenin gene targets. Lef/Tcf transcription factors bind within β-catenin’s Armadillo domain, which is entirely retained within β-Eng. Thus, as expected and demonstrated by way of coimmunoprecipitation, β-Eng binds the HMG family member, XTcf-3 (Fig. 6B). Such an interaction presumably facilitates the direct suppression of β-catenin/Wnt gene targets via the presentation of β-Eng’s C-terminal Engrailed repression domain to the basal transcription machinery. In support of this model, a dominant negative form of XTcf-3 mimics the activity of β-Eng by inhibiting endogenous dorsal axis formation within Xenopus embryos (Brannon et al., 1997).

Further evidence indicating that β-Eng’s activity is to actively repress gene transcription arises from the observation that β-Eng can translocate into the nucleus of early embryos expressing β-Eng MT mRNA (see Fig. 1 and text for further explanation of constructs). The experiment was performed three times, and each sample was collected in triplicate. Average fold induction from three experiments is shown (y axis), with error bars indicating ± one standard deviation from the mean recorded value.

Fig. 5. β-Eng suppresses transcription from a luciferase reporter containing the proximal promoter element of siamois. The graph represents normalized fold induction of transcription (levels measured in relative light units) of a luciferase reporter containing three Lef/Tcf binding sites (shaded boxes) over basal activation levels (1.00) of a control reporter, S0, containing mutated Lef/Tcf binding sites. Reporter DNA was injected into both blastomeres of 2-cell stage embryos. The S01234 reporter DNA was co-injected with 0.5 ng of β-eng mRNA (see Fig. 1 and text for further explanation of constructs). The experiment was performed three times, and each sample was collected in triplicate. Average fold induction from three experiments is shown (y axis), with error bars indicating ± one standard deviation from the mean recorded value.

Fig. 6. β-Eng is capable of entering the nucleus of early embryonic blastomeres and bind HMG box transcription factors. β-Eng can bind β-catenin’s native nuclear binding partner, XTcf-3, in vivo as shown by coimmunoprecipitation of both β-Eng and XTcf-3. In blastomeres of stage 9 Xenopus embryos, nuclei (nu) are stained red (A, left panel), indicating the presence of β-Eng MT protein as visualized by an alkaline phosphatase reaction, or APAAP (DAKO Inc.). In uninjected control embryos (A, right panel) treated with the same APAAP method, nuclei (nu) do not display a red stain, indicating that the APAAP reaction is not present. Nuclei from embryos injected with mRNA encoding the myc-epitope alone were also negative for nuclear staining (data not shown). (B) The nuclear activity of β-Eng is further supported by western analysis following coimmunoprecipitation of β-Eng with XTcf-3 (myc-blot, lane 3 and HA-blot, lane 1). Lysates of stage 10 embryos co-injected with mRNA encoding both β-Eng MT and XTcf-3/HA were precipitated with the anti-myc (9E10) and anti-HA (12CA5) antibodies, respectively. Co-injected samples are denoted by (+) and uninjected control samples are denoted by (−).
blastosomeres, as revealed by immunohistochemical techniques (Fig. 6A). This result was also expected because β-Eng retains a complete Armadillo repeat region capable of binding both the NLS-bearing Lef/Tcf factors (Huber et al., 1996), and nuclear pore components via NLS and importin-independent mechanisms (Fagotto et al., 1998). Collectively, the immunoprecipitation data and the nuclear translocation evidence strengthens the argument that β-Eng possesses a specific nuclear activity.

We have demonstrated that β-Eng suppresses β-catenin-dependent Wnt signaling, as shown by the morphological ventralization of Xenopus embryos in addition to β-Eng’s suppression of dorsal and organizer-specific molecular markers such as Xnr-3, siamois, gooseocoid and chordin. While both the signaling and adhesive function of β-catenin appear to be independent (Heasman et al., 1994; Peifer, 1995), selective suppression of β-catenin’s signaling function has previously been difficult to achieve. We expect that β-Eng will prove useful to various researchers working upon β-catenin-mediated Wnt signaling. For example, unpublished work in our laboratory includes use of β-Eng to inhibit β-catenin-dependent signaling in ventral Xenopus development and suggests that β-catenin may play a significant role in the patterning and formation of ventral structures. Unlike the use of antisense technology to deplete signaling pools of β-catenin within the entire embryo (Heasman et al., 1994), β-Eng may be expressed within discrete tissues or regions in the context of an otherwise normal embryo. Further, work in progress has demonstrated that β-Eng effectively suppresses β-catenin-mediated Wnt signaling in mammalian cells (canine MDCK kidney cells), and we thus expect that its tissue-specific expression will prove useful in a number of model animal systems, and perhaps even in the suppression of pathological Wnt signaling contributing to the progression of various carcinomas (Bullions and Levine, 1998; Miller et al., 1999; Peifer, 1997; Polakis, 1999).

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