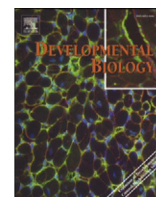
Developmental Biology 361 (2012) 220–231

Contents lists available at SciVerse ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/developmentalbiology



Xenopus Zic3 controls notochord and organizer development through suppression of the Wnt/β-catenin signaling pathway

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ARTICLE INFO

Article history:
Received for publication 12 April 2011
Revised 30 September 2011
Accepted 8 October 2011
Available online 26 October 2011

Keywords:
Zic
Xenopus laevis
β-Catenin
Spemann's organizer
Notochord

ABSTRACT

Zic3 controls neuroectodermal differentiation and left–right patterning in *Xenopus laevis* embryos. Here we demonstrate that Zic3 can suppress Wnt/β-catenin signaling and control development of the notochord and Spemann's organizer. When we overexpressed *Zic3* by injecting its RNA into the dorsal marginal zone of 2-cell-stage embryos, the embryos lost mesodermal dorsal midline structures and showed reduced expression of organizer markers (*Siamois* and *Goosecoid*) and a notochord marker (*Xnot*). Co-injection of *Siamois* RNA partially rescued the reduction of *Xnot* expression caused by Zic3 overexpression. Because the expression of *Siamois* in the organizer region is controlled by Wnt/β-catenin signaling, we subsequently examined the functional interaction between Zic3 and Wnt signaling. Co-injection of *Xenopus Zic* RNAs and *β-catenin* RNA with a reporter responsive to the Wnt/β-catenin cascade indicated that Zic1, Zic2, Zic3, Zic4, and Zic5 can all suppress β-catenin-mediated transcriptional activation. In addition, co-injection of *Zic3* RNA inhibited the secondary axis formation caused by ventral-side injection of β-catenin RNA in *Xenopus* embryos. Zic3-mediated Wnt/β-catenin signal suppression required the nuclear localization of Zic3, and involved the reduction of β-catenin nuclear transport and enhancement of β-catenin degradation. Furthermore, Zic3 co-precipitated with Tcf1 (a β-catenin co-factor) and XIC (I-mfa domain containing factor required for dorsoanterior development). The findings in this report produce a novel system for fine-tuning of Wnt/β-catenin signaling.

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Introduction

Zic proteins are zinc-finger-type transcription regulators that are widely conserved in eumetazons (Aruga et al., 2006; Ishiguro et al., 2007). In vertebrate embryos, Zic proteins are critical regulators of ectodermal development (Inoue et al., 2007b; 2008; Maurus and Harris, 2009; Sanek et al., 2009; Warr et al., 2008) (for earlier references, see Aruga, 2004; Merzdorf, 2007). They also play essential roles in the development of mesodermal components, as revealed in studies of *Zic* mutant mice (Aruga et al., 1999; Nagai et al., 2000; Purandare et al., 2002). Inoue et al. (2007a) revealed that the differentiation and proliferation of early mesodermal cells marked by *Wnt3a* expression were disturbed in *Zic2*/*Zic3* compound mutant mice, suggesting that Zic2 and Zic3 cooperatively control the development of mesoderm.

In *Xenopus laevis* embryos, there are five *Zic* genes (*Zic1–5*) that partly share spatiotemporal expression profiles and functions (Fujimi et al., 2006; Nakata et al., 1998; 2000), and *Zic1*, *Zic2* and *Zic3* are strongly expressed during gastrulation (Supplemental Figs. 1 and 2). Among them, Zic3 is the factor that links the BMP signal blockade and neuroectodermal differentiation (Aruga and Mikoshiba, 2011; Marchal et al., 2009; Nakata et al., 1997). However, *Zic3* is also involved in controlling mesodermal development and is expressed in the involuting mesoderm where *Bra* (also known as *Brachyury* or *Xbra*) is expressed (Kitaguchi et al., 2000; 2002). Bra is a T-box-containing transcription factor required for the early determination and differentiation of mesoderm (Showell et al., 2004) and is capable of inducing *Zic3* expression (Kitaguchi et al., 2002). In the early gastrula, *Zic3* expression in the mesoderm is strongest in the dorsal region, including the organizer (Spemann's organizer) region, but its expression diminishes in the late gastrula (Kitaguchi et al., 2000). Zic3 in the organizer region has been proposed to mediate the left–right axis coordination activities (Kitaguchi et al., 2000). However, there have been no reports that directly indicate the role of Zic3 in the development of the organizer.

The area of the organizer is thought to be defined by gradients of β-catenin (along the dorsal–ventral axis) and secreted signals from the vegetal area (along the animal–vegetal axis) at early stages (Clevers, 2006). Some genes that are required for organizer formation are downstream of the β-catenin signaling pathway. In the "signal-off" state, the cytosolic pool of β-catenin is bound to a destruction

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doi:10.1016/j.ydbio.2011.10.026

complex that includes Axin and APC; then, phosphorylation of β-catenin by GSK3β and CKIα leads to degradation via the ubiquitin–proteasome system. The dishevelled (Dsh) protein can free β-catenin from degradation, and β-catenin accumulates in the nucleus in the "signal-on" state. β-Catenin located in nucleus associates with the Tcf/Lef proteins and activates the expression of downstream target genes. As a nuclear component of the Wnt/β-catenin pathway, β-catenin regulates various genes important for the formation of organizer in dorsal mesoderm [*e.g.*, *Nr3*(*Xnr-3*), *Siamois*, and *Twn*] (Brannon et al., 1997; Clevers, 2006; Laurent et al., 1997; McKendry et al., 1997; Nusse, 2005a). Although Wnt signaling is required for the onset of organizer formation, it is subsequently suppressed by Wnt antagonists (*e.g.*, Dkk-1 and Frzb). The Wnt antagonists are required for the ability of intact dorsoanterior structures to inhibit the posteriorizing signals mediated by Wnt signals (Meinhardt, 2006), indicating that timely cessation of the Wnt signal is essential for dorsal axis formation.

In this study, we investigated the role of *Xenopus* Zic3 in organizer development, focusing on its inhibitory effect on β-catenin-dependent transcriptional activation. This study revealed a novel role for *Xenopus* Zic proteins and a novel mechanism for controlling Wnt/β-catenin signaling.

Materials and methods

Embryo manipulation

Adult *X. laevis* were purchased from Watanabe Zoushoku (Hyogo, Japan). Embryos were obtained from human chorionic gonadotrophin (hCG)-injected pigmented females by *in vitro* fertilization. The jelly coats were removed by immersing the embryos in 1% sodium mercaptoacetate (pH 9.0) for a few minutes. Embryos were cultured in 0.1× Steinberg's solution and staged according to Nieuwkoop and Faber(1967). Microinjection was carried out as previously described (Moon et al., 2002).

Cell lines

COS7 (Immunoblot and Immunoprecipitation) cells were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS). Transfection of plasmid vectors to the cells was performed with *Trans*IT®-LT1 (Mirus Bio LLC., Madison, WI, USA) according to the manufacturer's instruction.

Whole mount in situ hybridization and histochemical analysis

The embryos were cultured until several stages (St. 9.5–33). Whole-mount *in situ* hybridization was performed as described (Shain and Zuber, 1996) with a slight modification: proteinase K (10 μg/mL) treatment was extended to 5 min to enhance the signal. Probe sequences for *HoxB9*, *Bra*, *Gooscoid*, *Xnot*, *β-catenin*, *Zic1*, *Zic2*, and *Zic3* are available upon request. For paraffin sectioning, embryos were fixed with MEMFA (Sive et al., 2000) for 1 h and kept in methanol at −20 °C. Before paraffin embedding, the samples were transferred to butanol and embedded in Paraplast X-tra (Fisher Scientific, Suwanee, GA, USA). Embryos were sectioned at 10-μm thickness and stained with hematoxylin and eosin.

Plasmids and morpholino oligos

The Zic1–5 expression vectors were described previously (Fujimi et al., 2006; Nakata et al., 1997, 1998, 2000). Zic3-ZF and Zic3ΔN correspond to XZ3d7 and XZ3d4 (Kitaguchi et al., 2000), respectively. pCS2 expression vectors (Turner and Weintraub, 1994) for wild type (WT) human ZIC3 and its mutants ZIC3-W255G were described (Chhin et al., 2007), and those for ZIC3-P217A, ZIC3-T323M, ZIC3-K405E, and *Xenopus* Zic2-R428P [equivalent to human ZIC2-R409P in (Hatayama et al., 2011)] were generated using QuickChangeII site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA). Expression vectors for Bra, Siamois, Nr3, β-catenin, XIC, Tcf1, and Tcf3 were generated by PCR amplification of the protein coding regions from *Xenopus* embryo cDNA and subsequent cloning into pCS2 vectors that were modified to have Myc, Flag, or hemagglutinin (HA)-epitope tags. TOPFLASH and FOPFLASH luciferase reporters were purchased from Upstate Biotechnology Inc. (Charlottesville, VA, USA). Morpholino oligonucleotides (MOs) for *Zic2* (5′-ACTGGGGACCAGCGTCTAGTAGCAT-3′), *Zic3* (5′-TCCTCCATCTAATAGCATTGTCATG-3′) and a standard control morpholino oligo (as a negative control) were purchased from Gene Tools, LLC. (Philomath, OR, USA). We confirmed that the Standard Control Oligo had no effects on Wnt signaling or Zic function (data not shown).

RT-PCR analysis

RNAs or MOs were injected into the dorsal marginal zone of embryos at the 4-cell stage. To obtain the mRNA samples for RT-PCR, the dorsal mesoderm area of early-gastrula embryos was dissected with 2 pairs of tweezers at stage 10.5. From 5 pieces of dorsal mesoderm, total RNA was extracted as a sample. Primer sets for detection of *Bra* and *ODC* were as listed on De Robertis's Laboratory Home Page (http://www.hhmi.ucla.edu/derobertis/index.html). Primer sets for *Siamois* and *Nr3* were as described (Domingos et al., 2001). Other primer sets were *Twn* (F: 5′-TCCTGTGTTCTGCCCACCA-3′ and R: 5′-CTGTTGGGTGCCGATGGTA-3′) and *β-catenin* (F: 5′-GCAGTTGCTGTATTCGCCTATTGAA-3′ and R: 5′-TACAAGTCAGTGTCAAACCAGGCCAGT-3′). The indicated results are representative of four independent experiments showing similar tendency.

Secondary axis formation assay

RNAs or MOs were injected into the ventral marginal zone of one cell at the 4-cell stage. Injected embryos were cultured until the tailbud stage (stage 33) and fixed with MEMFA. After the fixation, the embryos were washed in phosphate buffer saline without salt [PBS(−)] for binocular microscopy observation. We defined embryos with obvious double-headed structures as having second axis formation. More than 40 embryos were assessed for each injection. The second axis frequencies are those from a single trial out of three independent trials that showed reproducible results. All embryos in one trial were siblings.

Luciferase reporter gene assay

For the reporter assay, DNA and RNAs were co-injected into the animal pole regions of 2 blastomeres at the 2-cell stage. Five injected whole embryos at stages 10–10.5 were used for a sample of luciferase reporter assay. Three samples were tested for one condition ($n = 3$). Luciferase activity was measured in accordance with the manufacturer's recommendations (Luciferase Assay System, Promega, Madison, WI, USA) using a Minilumat LB 9506 luminometer (Berthold, Bad Wildbad, Germany). Measured luciferase activity was normalized by the protein amount in the sample lysate. Means and standard deviations of 3 independent samples are shown.

Immunohistochemistry

Immunohistochemical staining of *Xenopus* embryos was performed as described previously (Fagotto and Brown, 2008). Alternate serial sections of stage-10.5 embryos were stained with an anti-β-catenin antibody [raised against the C-terminus (amino acids 680–781) of human β-catenin; H-102, Santa Cruz, 1:100] or anti-β-galactosidase antibody (Cappel, 1:500). Images were taken with a confocal microscope (FV1000, Olympus, Tokyo, Japan) and analyzed by using ImageJ software (http://rsbweb.nih.gov/ij/). Images of nuclear-localized β-catenin images were obtained as follows. First, a binary image of the nucleus

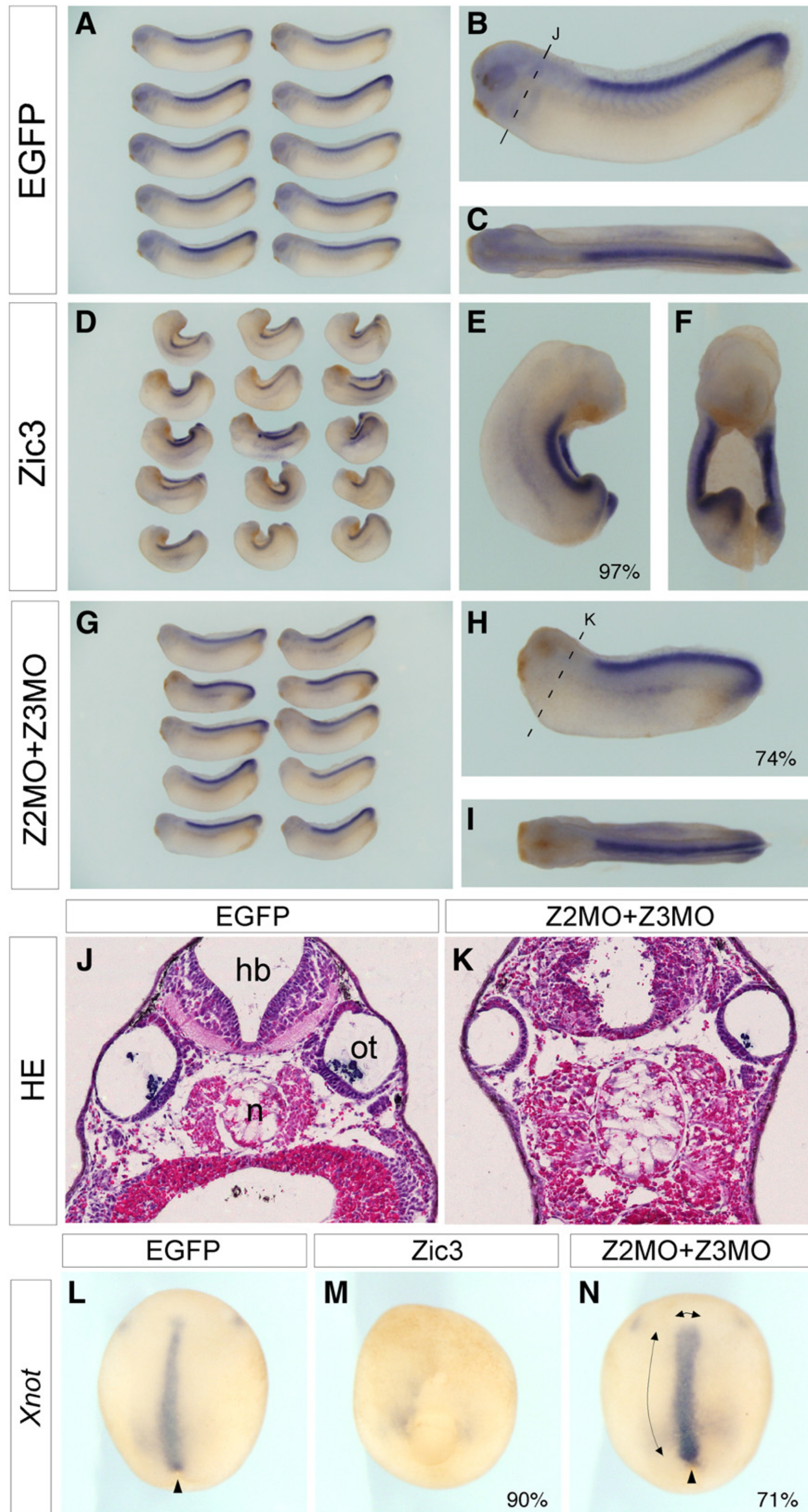

Fig. 1. *Zic3* has a role in notochord development. *EGFP* RNA alone (A–C, J, L; *EGFP*, 400 pg) or *EGFP* RNA (400 pg) with *Myc-tagged Zic3* RNA (D–F, M; *Zic3*, 200 pg) or Zic morpholino oligos (G–I, K, N; *Z2MO + Z3MO*, Zic2MO and Zic3MO, 30 ng each) were injected into the dorsal marginal zone of the two dorsal blastomeres of embryos at the 4-cell stage. The embryos were fixed at the tailbud stage (A–K, stage 33) or early neurula stage (L–N, stage 14). In panels A–I, expression of the posterior neural marker *HoxB9* was analyzed by whole-mount *in situ* hybridization (WMISH; lateral view is shown in A, B, D, E, G, and H, and dorsal view is shown in C, F, and I). Panels J and K are hematoxylin-and-eosin-stained transverse sections of the embryos shown in panels B and H respectively (*dashed lines* indicate the section planes). n, notochord; hb, hindbrain; ot, otic vesicle. In panels L–N, expression of the notochord marker *Xnot* was analyzed by WMISH (n = 35–39). Images show dorsal views of the embryos. The indicated percentages of embryos had phenotypes similar to those shown in the images. *Arrowheads*, blastopores; *long and short arrows*, the rostrocaudal and left–right extent of *Xnot* expression.

was created from DAPI-stained images by setting an appropriate threshold. Second, the β-catenin in the cell nuclei area was selected by using the image calculation method "AND" between "β-catenin image" and "nuclear binary image". Third, the nuclear β-catenin signals were measured in regions that overlapped the β-galactosidase-expressing organizer area in the adjacent sections.

Immunoblot and immunoprecipitation

Injected embryos (25 embryos) or cultured cells (1×10^6 cells) were homogenized with 3.5 mL (embryo) or 1 mL (cell) of immunoprecipitation buffer [50 mM Tris–HCl (pH 8.0), 150 mM NaCl, 0.1% (w/v) sodium dodecyl sulfate (SDS), 0.3% (w/v) deoxycholate sodium salt (DOC), 0.5% Triton X-100, 50 mM NaF, 1 mM Na_3VO_4, 0.1 mM dithiothreitol (DTT), 10 μg/mL leupeptin, 1 mM phenylmethanesulfonyl fluoride (PMSF), 10 mM MG132] containing Complete Protease Inhibitor Cocktail (Roche, Basel, Switzerland). After centrifugation (30,000×g, 4 °C, 30 min), the supernatant was transferred to a new tube and pre-cleaned with Protein G agarose beads (Thermo Scientific Pierce, Rockford, IL, USA). Aliquots of the sample were incubated with the antibodies (~4 μg) for 60 min at 4 °C, followed by incubation with Protein G agarose beads (4 °C, 90 min) and a wash with immunoprecipitation buffer. The precipitated proteins were detected by immunoblotting using an ECL detection system (GE Healthcare, Piscataway, NJ, USA). Antibodies against pan Zic (Inoue et al., 2007b), actin (A2066, Sigma), β-catenin (H-102, Santa Cruz), Myc-tag, (9E10, Santa Cruz), and Flag-tag (M-2, Sigma) were used.

Results

Zic3 overexpression in the dorsal marginal region causes organizer defects

To address the role of *Xenopus* Zic3 in the organizer region, we injected *Zic3* RNA into the dorsal marginal zone at the 4-cell stage. At the tailbud stage, the injected embryos showed a short anterior posterior (A–P) axis, neural tube closure defects, and severe dorsal curvature (Figs. 1D, E, and F). Expression of the posterior neural marker *HoxB9* was not impaired compared to control embryos (Figs. 1A, B, and C). This result indicates that the abnormality was not primarily due to impaired neuroectodermal differentiation or posteriorization of the neural tube. At the late gastrula stage, we did not observe expression of *Xnot*, an early notochord marker, in the dorsal midline of embryos injected with *Zic3* RNA, whereas we did observe this pattern in the control embryo (Figs. 1L and M), indicating that an impairment of notochord development underlies the

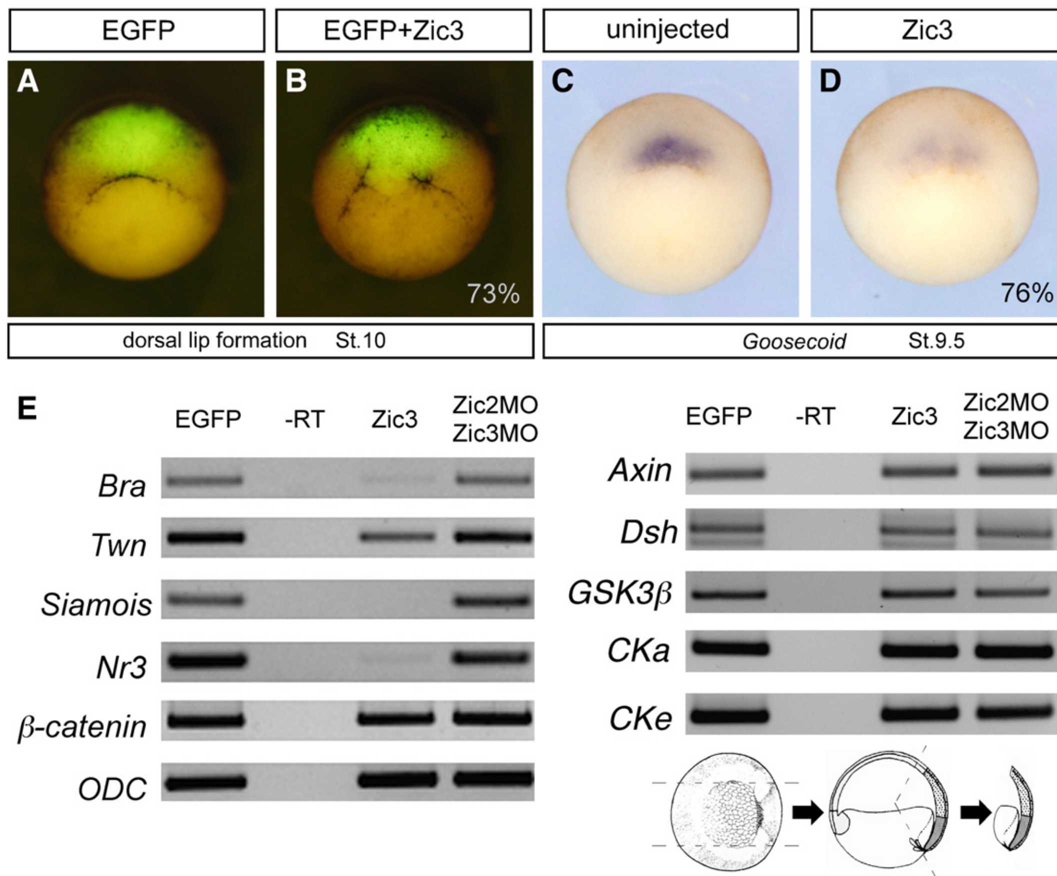

Fig. 2. Organizer formation is impaired by *Zic3* overexpression. *EGFP* RNA alone (A; *EGFP*, 400 pg), *EGFP* RNA (400 pg) with *Myc-tagged Zic3* RNA (200 pg) (B; *EGFP+Zic3*), or *Myc-tagged Zic3* RNA alone (200 pg) (D; *Zic3*) were injected into the dorsal marginal zone of the dorsal two blastomeres of embryos at the 4-cell stage. The injected embryos and an uninjected sibling embryo (C) were observed at stage 10 (A, B) or stage 9.5 (C, D). (A, B) Green EGFP signals derive from the injected RNA. Dorsal lips were frequently defective in embryos with *Zic3* RNA injection (73%, n = 16). (C, D) Expression of an organizer marker gene (*Goosecoid*) was analyzed by WMISH. Reduction of *Goosecoid* expression was frequently observed in the Zic3 RNA-injected embryos (76%, n = 27). (E) RT-PCR analysis. EGFP RNA alone (*EGFP*, 400 pg), EGFP RNA (400 pg) with Myc-tagged Zic3 RNA (200 pg) (*Zic3*), or EGFP RNA (400 pg) with Zic morpholino oligos (30 ng each) (*Zic2MO + Zic3MO*) was injected into the dorsal marginal zone of the dorsal two blastomeres of embryos at the 4-cell stage. At stage 10.5, the dorsal part was dissected as in bottom cartoon and used for total RNA extraction. Direct targets of β-catenin (*Bra*, *Twn*, *Siamois*, and *Nr3*) were downregulated by the Zic3 RNA injection, whereas genes in the Wnt/β-catenin signaling cascade (*β-catenin*, *Axin*, *Dsh*, *GSK3β*, *CKα* and *CKε*) and a housekeeping gene (*ODC*) were not changed. The cDNA synthesis reaction for the sample injected with EGFP RNA alone was also performed without reverse transcriptase (*-RT*) to confirm the absence of chromosomal DNA contamination.

later abnormalities. Earlier, at mid-gastrulation, dorsal lip formation was disturbed (Figs. 2A and B), suggesting that the properties of the organizer region were altered. Accordingly, expression of *Goosecoid*, an organizer marker, was substantially reduced in the early gastrula [Stage (St.) 9.5; Figs. 2C and D]. Collectively, these results indicate that *Zic3* overexpression in the dorsomarginal region alters the properties of the organizer and impairs notochord development.

We next performed a gene function knockdown. Because Zic2 and Zic3 are known to cooperatively control early mesodermal development in mice (Inoue et al., 2007a), we tested the effect of injecting morpholino oligonucleotides (MOs) targeting these proteins into the dorsal marginal region. The injection of *Zic3* MO or *Zic2* MO alone caused marginal abnormalities (data not shown). However, the combined injection of *Zic2* and *Zic3* MOs resulted in wider and thicker *Xnot* expression along the entire A–P axis in the late gastrulae (Figs. 1L and N). Notably, the anterior notochord thinning seen in the control embryo was not found, and the notochord remained thick with a blunt anterior end at St. 14 (Fig. 1N). Later, at St. 33, the *Zic2*/*Zic3* knockdown embryos showed a hyperplastic notochord (Figs. 1J and K), A–P axis shortening, and reduced head structures (Fig. 1G–I). Both the overexpression and knockdown phenotypes support the idea that Zic3 negatively controls the development of the notochord.

Zic3 can inhibit Wnt/β-catenin signaling

Wnt/β-catenin signaling has critical roles in the development of the organizer and notochord (Chu et al., 2007; Meinhardt, 2006). We next tested whether the downstream targets of Wnt/β-catenin signaling are affected by *Zic3* overexpression. For this purpose, we performed an RT-PCR assay of the dorsal lip tissues from St. 10.5 *Zic3*-overexpressing embryos. Among the β-catenin downstream targets, *Bra*, *Siamois*, and *Nr3* were strongly downregulated by the overexpression, and *Twn* also tended to be decreased (Fig. 2E). But the mRNA levels of the genes constituting the Wnt/β-catenin signaling cascade (*β-catenin*, *Axin*, *Dsh*, *GSK3β*, *CKe*, and *CKa*) were not affected by either overexpression or knockdown (Fig. 2E).

Among the target genes downstream of Wnt/β-catenin signaling, *Siamois* is essential for β-catenin-mediated formation of the organizer (Carnac et al., 1996; Fan and Sokol, 1997; Kessler, 1997). *Siamois* expression is directly regulated by Tcfs (Fan et al., 1998), and Siamois inhibition blocks axis development (Fan and Sokol, 1997; Kessler, 1997). We therefore tested whether co-injection of *Siamois* RNA with *Zic3* RNA rescues the defect caused by *Zic3* overexpression. *Siamois* co-expression reversed the disappearance of the *Xnot* expression in the dorsal region of mid-gastrula embryos (Fig. 3A) to a level comparable to that seen in embryos injected with a control RNA (β-galactosidase) (Fig. 3B). Although another downstream target, *Bra*, did not rescue the phenotype, *Nr3* provided intermediate rescue activity (Fig. 3B). However, we did not see the complete rescue of the *Zic3* overexpression later phenotypes such as short A–P axis in embryos co-injected with either *Zic3*/*Siamois* or *Zic3*/*Nr3* RNAs (data not shown). These results suggest that Zic3 inhibits the Wnt/β-catenin signaling pathway, and the Wnt-signaling inhibition partly accounts for the developmental defect in the notochord caused by *Zic3* overexpression.

We attempted to further confirm the suppression of β-catenin signaling by Zic3. Overexpression of β-catenin in the ventral side of *Xenopus* embryos results in the formation of secondary axes (Funayama et al., 1995). When we injected *β-catenin* RNA into the ventral marginal zone, we found a secondary axis which was never seen in uninjected animals (Fig. 4A) in 78% of the embryos (Fig. 4B). The appearance of secondary axes was reduced to 4% by the addition of *Zic3* RNA to the *β-catenin* RNA (Fig. 4C). The reduction was lessened to 70% by the further addition of a *Zic3* MO (Fig. 4E). No secondary axes were observed with a single injection of *Zic3* MO (Fig. 4D) or *Zic3* RNA (Fig. 4F). Taking these data together, we conclude that Zic3 can inhibit the activity of β-catenin for forming a secondary axis.

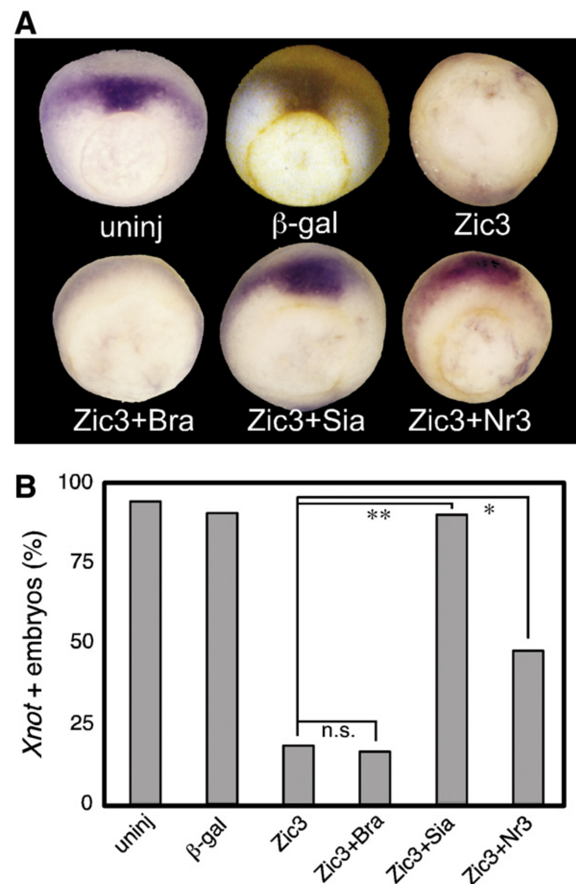

Fig. 3. Rescue of Zic3-attenuated notochord progenitor marker expression by co-injection of Siamois RNA. (A) *Xnot* expression in early gastrula. Vegetal views of the embryos with dorsal at the top. The embryos were injected with the RNAs indicated below (1000 pg each). *Xnot* expression was detected in the dorsal area adjacent to the yolk plug (purple signal) in the uninjected (*uninj*) embryo and the β-galactosidase (*β-gal*)-injected embryo, but disappeared in the *Zic3* RNA (*Zic3*)-injected embryo. β-gal, Zic3, Bra, Sia and Nr3 mean the injected RNAs of *β-galactosidase*, *Zic3*, *Bra*, *Siamois* and *Nr3*, respectively. (B) Percentages of embryos with *Xnot* expression. Co-injection of *Siamois* fully restored normal *Xnot* expression. *$P<0.01$; **$P<0.001$; n.s., $P>0.05$ in comparison to *Zic3* alone injection in χ^2 test. More than 50 embryos were analyzed in each group.

Zic3 blocks β-catenin-mediated transcriptional activation

To confirm the Zic3-mediated inhibition of Wnt/β-catenin signaling, we injected *Zic3* RNA along with *β-catenin* RNA and a Wnt/β-catenin signaling reporter vector (TOPFLASH vector, Kim et al., 2000) into the animal pole at the 2-cell stage. The luciferase reporter activity was increased by the presence of *β-catenin* RNA (Fig. 5A). As expected, co-injection of *Zic3* RNA reduced the reporter gene expression. Control experiments for this reporter assay (the FOPFLASH vector and *Zic3* MO co-injection) indicated that this system was specific for β-catenin transactivation (Figs. 5A and B). These experiments provided further evidence that Zic3 inhibits β-catenin-dependent transcription activation.

We next tested the effect of overexpression of Zic1, Zic2, Zic4, or Zic5 on β-catenin-dependent transcription activation, and found that all were able to suppress the β-catenin-mediated reporter activation (Fig. 5C). Then we evaluated the effects of Zic3 or Zic2 mutations equivalent to those found in congenital heart anomaly/heterotaxy patients (Chhin et al., 2007; Hatayama et al., 2008; Ware et al., 2004) or a schizophrenia patient (Hatayama et al., 2011) because these mutations are known to variously impair the nuclear localization and transcriptional activation properties. In terms of nuclear localization, highly defective mutants (ZIC3-T323M, ZIC3-W255G)

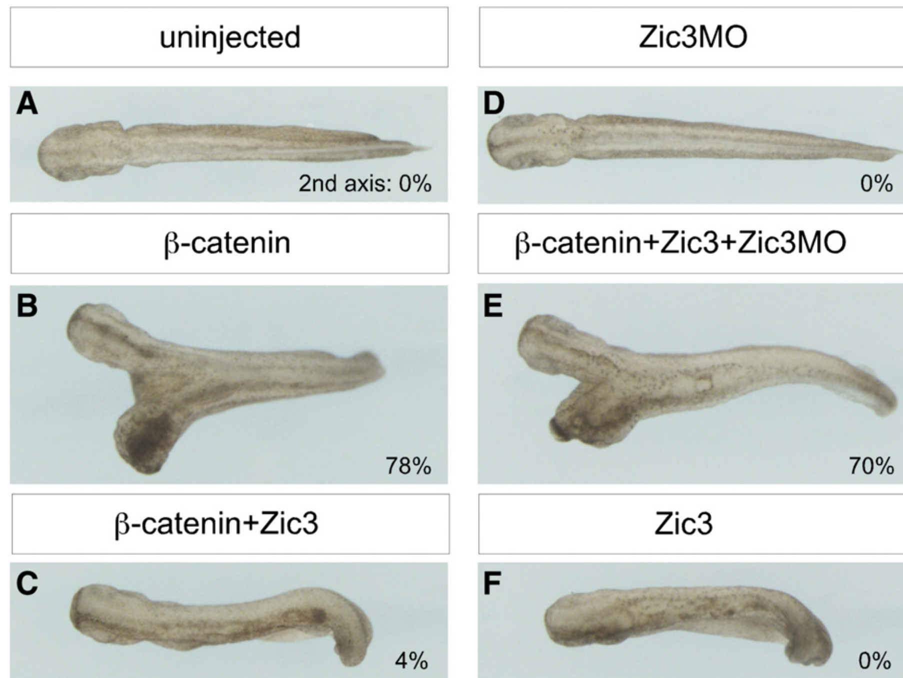

Fig. 4. Zic3 inhibits secondary axis formation induced by ectopic β-catenin. Typical shapes of the tailbud stage embryos (stage 33, dorsal view) subjected to the second axis formation assay are shown. Percentages given below indicate the embryos with secondary axis among 43–50 embryos for each experimental group. Flag-tagged β-catenin RNA (200 pg) injected into the ventral marginal zone at the 4-cell stage frequently induced the secondary axis structure (B; *β-catenin*, 78%). The secondary head structure was rarely observed after co-injection of Flag-tagged β-catenin RNA (200 pg) and Zic3 RNA (50 pg) (C; *β-catenin + Zic3*, 4%). The suppression of the secondary axis effect by Zic3 RNA was abolished by Zic3MO (10 ng) (E; *β-catenin + Zic3 + Zic3MO*, 70%). There were no embryos with ectopic heads in Zic3MO-injected (D; *Zic3MO*, 0%), Zic3 RNA-injected (F; *Zic3*, 0%), or control embryos without injection (A; *uninjected*, 0%).

lacked the ability to suppress β-catenin-dependent transcription activation whereas the mutant of normal nuclear localization (ZIC3-P217A) suppressed it as strongly as WT, and a mutant of mildly defective nuclear localization (ZIC3-K405E) showed partial suppression (Fig. 5D). Thus, the degree of nuclear localization correlated with the suppression activity. On the other hand, transcriptional activation capacity was not correlated with the suppression activity as seen in Zic2-R428P (Fig. 5D). These results suggested that the presence of Zics in the nucleus is sufficient for reducing β-catenin signaling and that Zic activity as transcriptional activity is not needed.

In the course of Wnt/β-catenin signal activation, β-catenin translocates from the cytoplasmic compartment to the cell nucleus for the transcriptional activation of the target genes (Clevers, 2006). Consistent with this, β-catenin accumulates in the cell nuclei of the organizer-forming region where Wnt signaling is thought to be activated (Schohl and Fagotto, 2002). We therefore assessed the effects of Zic3 overexpression on endogenous β-catenin nuclear accumulation *in vivo*. For this purpose, we injected *Zic3* RNA and control β-galactosidase RNA, or β-galactosidase RNA alone, into the dorsal marginal region of 2-cell-stage embryos and examined the extent of β-catenin nuclear accumulation in the β-galactosidase-positive organizer region by immunofluorescence staining. The analysis revealed that the β-catenin nuclear accumulation with *Zic3* overexpression was about two thirds of that with β-galactosidase expression alone (Figs. 6A and B). Thus, Zic3 overexpression resulted in the decrement of nuclear β-catenin.

We next tested the interaction of Zic3 with β-catenin in immunoprecipitation experiments using lysates of tissues injected with Zic3 and β-catenin RNAs. However, we did not detect a significant physical interaction (data not shown). Instead, both anti-Flag epitope antibody and anti-β-catenin C-terminus antibody detected lower molecular weight bands than those of N-terminally Flag-tagged β-catenin only when Zic3 was coexpressed (Fig. 6C). The bands seemed to be β-catenin degradation products, suggesting that Zic3 overexpression enhances β-catenin degradation.

Zic3 physically interacts with Tcfs, and the association is enhanced by XIC

We next tested the interaction of Zic3 with Tcfs, binding partners for β-catenin in cell nuclei. We carried out immunoprecipitation using Zic3 epitope-tagged at the N-terminus and lysates of Tcf1-transfected mammalian cells. Tcf1 coprecipitated Zic3, and Zic3 coprecipitated Tcf1 (Fig. 7). This interaction was also seen with another Tcf family member, Tcf3 (data not shown).

In the course of the immunoprecipitation experiments, we noticed that I-mfa family proteins (XIC or I-mfa) bind both Zic3 and Tcf3 and functionally inhibit them (Mizugishi et al., 2004; Snider et al., 2001). XIC is required for Siamois activity and dorsoanterior development in *Xenopus* embryos (Snider and Tapscott, 2005). We therefore tested the interactions among Zic3, Tcf1, and XIC in the same assay system. We observed Zic3–XIC and Tcf1–XIC interactions, as expected (Fig. 7). Interestingly, the presence of XIC increased the Zic3–Tcf1 association as evidenced by the increased amount of precipitation (Fig. 7). The result raises the possibility that the interaction between Zic3 and Tcf1 interferes with the transcriptional activation by β-catenin. However, our immunoprecipitation experiments using *Xenopus* embryo did not support that idea (data not shown). There were decrements in the Zic3, Tcf1 and XIC bands in the input immunoblot, suggesting that the complex formation affected the stability or subcellular localization of each protein.

Discussion

The role of Zic3 in organizer and notochord development

We show here that Zic3, which controls neuroectodermal differentiation and left–right patterning, also plays a role in the development of the organizer and notochord. In late blastula, moderate *Zic3* expression can be detected in the dorsal marginal zone (Supplemental Fig. 1). In the gastrula, stronger *Zic3* expression appears in the prospective neural

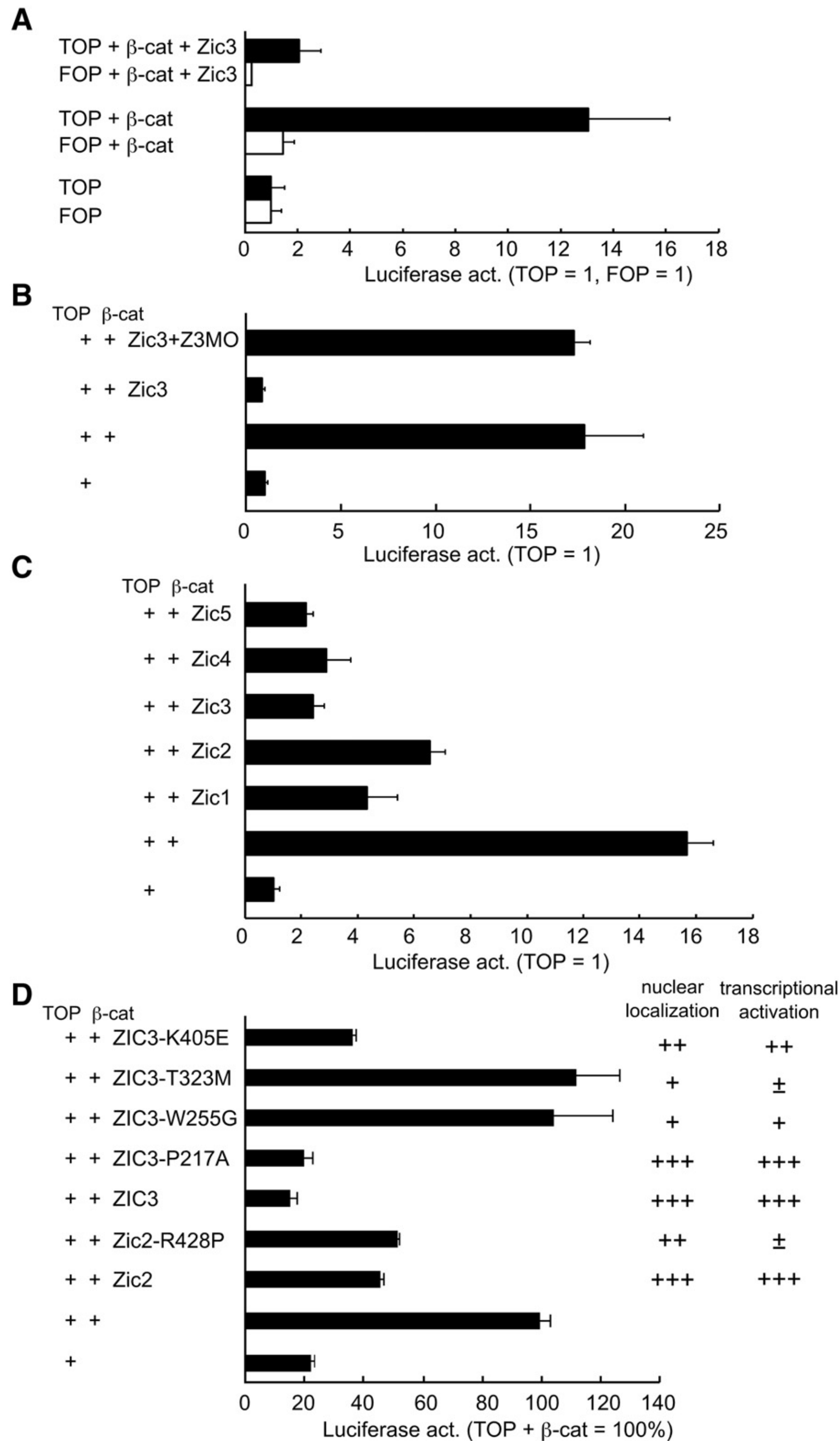

Fig. 5. TOPFLASH reporter gene assay in *Xenopus* gastrula embryos. The TOPFLASH reporter vector (*TOP*, 200 pg), FOPFLASH vector (*FOP*, 200 pg), Flag-tagged β-catenin RNA (*β-cateninFT*, 300 ng), β-catenin RNA (*β-catenin*, 300 ng), Myc-tagged Zic RNA (*Zic1–Zic5*, 200 pg each), and/or Zic3 were injected into animal pole regions of both blastomeres of 2-cell-stage embryos in the indicated combinations. The TOPFLASH reporter vector contains Tcf-binding sites upstream of the promoter region, whereas the Tcf-binding sites are mutated in the control FOPFLASH vector. Embryos were collected at stage 10.5 in pools of 5 embryos, and assayed for luciferase activity in triplicate (total of 15 injected embryos for each value). The activity was normalized by the protein amount in the sample lysate. Mean luciferase activities of embryos injected with the TOP or FOP reporter vector only are defined as one relative luciferase unit (RLU) in each experiment. Error bars indicate standard deviation. (A) Co-injected β-catenin increased luciferase activity in the presence of the TOP reporter (*TOP + β-cat*). The increase was attenuated by Zic3 RNA co-injection (*TOP + β-cat + Zic3*). (B) This inhibition was rescued by co-injection of Zic3MO (*Z3MO*). (C) Comparison among the *Xenopus* Zic family members for the β-catenin-suppressing activities. (D) The effects of nuclear localization extent and transcriptional activation abilities of Zic3 or Zic2 mutants. Approximate nuclear localization extents and transcriptional activation abilities are indicated as follows: +++, ≥100; ++, 80–60; +, 50–30; ±, ≤20; where values of wild type Zic2 or Zic3 are 100. The values are based on previous studies [ZIC3-P217A, ZIC3-T323M, and ZIC3-K405E (Ware et al., 2004); ZIC3-W255G (Hatayama et al., 2008); Zic2-R428P (Hatayama et al., 2011)].

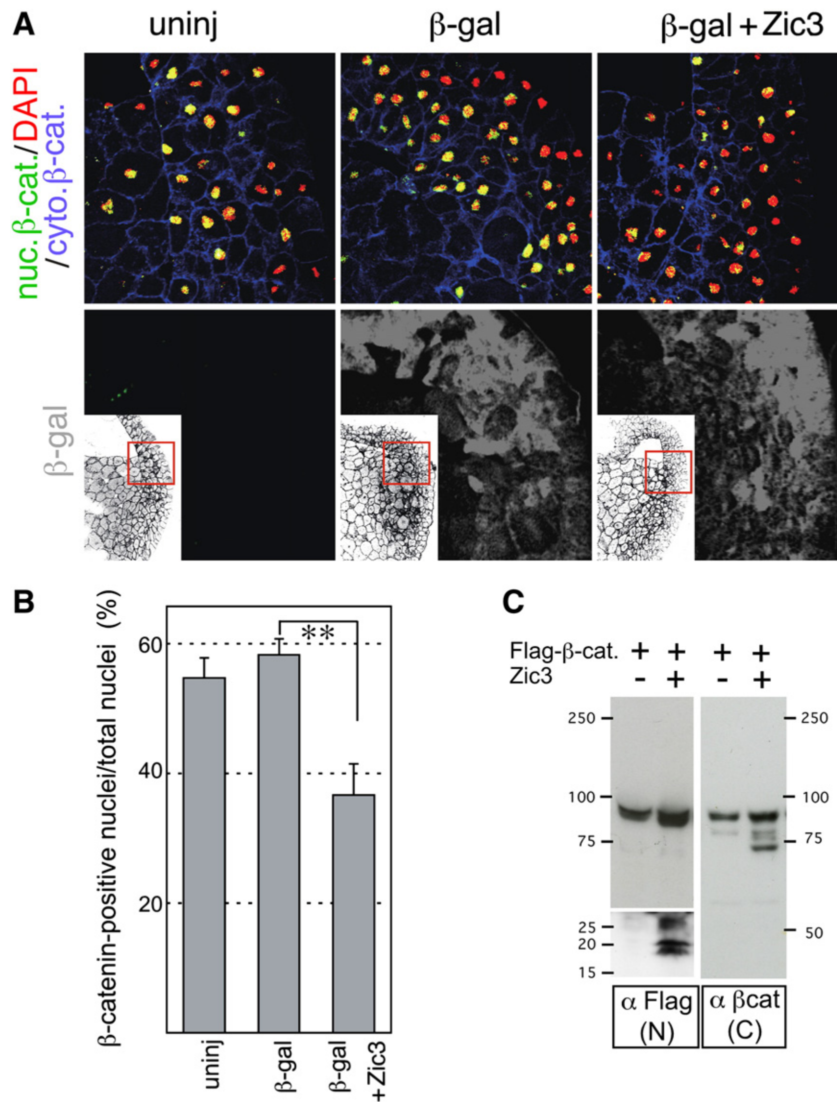

Fig. 6. β-catenin nuclear localization is impaired by *Zic3* expression in gastrula embryos. (A) Representative images for β-catenin immunofluorescence staining. Neighboring sections were stained with anti-β-catenin and anti-β-galactosidase (*β-gal*) antibodies. In each image, the animal pole is at the top and the dorsal side is to the right. Upper images show the β-catenin signal (nuclear, *green*; non-nuclear, *blue*) and DAPI signal (nuclear, *red*), and lower images show the β-galactosidase signal (*gray*) of the injected area. The *yellow* indicates the overlapping of nuclear β-catenin and DAPI signals. The *insets* are low magnifications in which the boxed regions indicate the stained regions. (B) The graph shows percentages of the β-catenin/DAPI double-positive cell nuclei area among the total DAPI-stained cell nuclei in the organizer region. Nuclear transported β-catenin was significantly lower in Zic3-injected embryos. $^{**}P<0.01$, Kruskal–Wallis test followed by Steel–Dwass post hoc test. Error bars, standard error of the mean. (C) Immunoblotting analysis for β-catenin. Embryos were injected with the β-catenin or (β-catenin and Zic3) RNA. Immunoblotting of the embryo tissue lysate using an antibody against the anti-β-catenin C-terminus (*β-cat.*) or an antibody against the Flag-tag located on the N-terminus of β-catenin (*Flag*). Zic3 RNA co-injection enhanced the degradation of β-catenin.

plate region, while expression remains moderate in the involuting mesoderm, including notochord progenitors (Kitaguchi et al., 2000) (Supplemental Fig. 1). Therefore, the interaction of Zic3 with the Wnt/β-catenin signal could influence both the formation of the organizer and subsequent differentiation of the notochord from the organizer. Although *Zic3* overexpression can inhibit organizer formation, our knockdown experiments only showed abnormalities in the notochord precursor cells. In this regard, we propose that the inhibition of the Wnt/β-catenin signal by Zic3 has biological meaning mainly in the regulation of notochord formation. β-Catenin is necessary for maintaining the competency of notochord progenitor cells for notochord differentiation in both *Xenopus* (Chu et al., 2007) and mouse (Ukita et al., 2009). In addition, Wnt signaling is strongly active in the notochord progenitor cells in mice (Ukita et al., 2009). Therefore, Zic3 in the notochord progenitor cells likely has a role in tuning Wnt/β-catenin signaling strength in the cells.

Zic2 and Zic3 are likely to share a common role in notochord development, as indicated by the synergistic effect of their knockdown. Accordingly, *Zic2* expression can also be detected in the dorsal involuting mesoderm (Supplemental Fig. 1). Furthermore, Zic2 also had a suppressive effect in the TOPFLASH reporter assay (Figs. 5C and D), suggesting that Zic2 inhibits the Wnt/β-catenin signaling pathway in the course of notochord development. The cooperativity of Zic3 and Zic2 has been also indicated through the analysis of the *Zic2*/*Zic3* compound mutant mice (Inoue et al., 2007a).

Although the knockdown experiments in this study did not clearly demonstrate the involvement of Zic3 or Zic2 in organizer formation, the involvement of Zic3 in organizer formation might still be possible. This is because RNA injection into *Xenopus* embryo at cleavage stage may be too late to affect the maternally controlled Wnt/β-catenin signaling for organizer formation (Tao et al., 2005). Taken together with the results that both *Zic2* and *Zic3* are expressed before gastrulation and the attenuation of *Xnot* expression induced by *Zic3* overexpression is partly recovered by co-overexpression of *Siamois*, we speculate that Zic3 and Zic2 could exert their roles in proper organizer formation through fine tuning of Wnt/β-catenin signaling output.

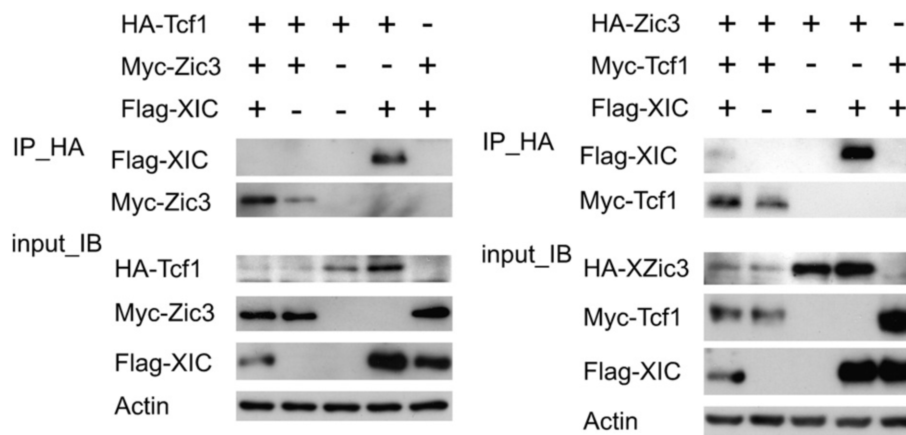

Fig. 7. Zic3 can physically interact with Tcf1 and XIC. Immunoprecipitation experiments indicating the physical interaction among Zic3, Tcf1, and XIC. Epitope-tagged *Xenopus* Tcf1, Zic3, and XIC were expressed in COS7 cells in the indicated combinations. Following immunoprecipitation (*IP*) with an anti-HA-tag antibody (*IP_HA*), the co-precipitated proteins were detected with an anti-Myc-tag antibody. The cell lysates (*input*) used for the immunoprecipitation experiments were also subjected to an immunoblot analysis (*input_IB*) using the antibodies against the epitope tags (HA, Myc, and Flag) and actin. Both the Tcf1 (*left*) and Zic3 (*right*) pull-down experiments suggested that Zic3 and Tcf1 physically interacted, and that this interaction was enhanced by XIC. XIC was co-precipitated with both Zic3 and Tcf1.

Besides the organizer formation and development, the biological roles of Zic–Wnt/β-catenin regulatory relationship can be postulated based on the expression patterns and known functions of Zic and Wnt/β-catenin signaling. First, higher expression of *Zic3* in the neuroectoderm may indicate differential Wnt/β-catenin signaling responses between mesoderm and neuroectoderm, because these two tissues are located close to each other and are exposed to Wnt ligands. Second, the interaction between Zic and Wnt/β-catenin signaling may be involved in the anterior–posterior patterning of the neuroectoderm. The Wnt/β-catenin pathway posteriorizes neural tissue (Domingos et al., 2001), whereas Zic3 promotes the differentiation of tissue into anterior neuroectoderm (Nakata et al., 1997). Third, the Zic–Wnt/β-catenin interaction could control the head-induction process, because some Wnt-antagonizing factors are known to have head-inducing activity (Bouwmeester et al., 1996; Glinka et al., 1998; Piccolo et al., 1999). This idea is supported by the result of the *Zic2*/*Zic3* double-knockdown experiment, which resulted in a reduction of head size (Fig. 1G).

The role of *Zic* genes in notochord development may be conserved in chordates. In mice, the tail deformities and axial shortening appears in *Zic2* knockdown, *Zic3* null and their compound mutants (Inoue et al., 2007a; Klootwijk et al., 2000; Nagai et al., 2000; Purandare et al., 2002), and the *Bra* expression in early mesoderm is reduced in *Zic2*/*Zic3* compound mutant (Inoue et al., 2007a). In ascidians, *ZicL* or *HrzicN* knockdown results in the impaired differentiation of the notochord precursor cells with the loss of *Bra* expression (Imai et al., 2002; Wada and Saiga, 2002; Yagi et al., 2004). Furthermore, it should be noted that the overexpression of *HrZicN* resulted in loss or reduction of *Bra* expression (Wada and Saiga, 2002). Together with our result that *Bra* is down-regulated by Zic3 overexpression in the dorsal marginal zone (Fig. 2E) where the Wnt/β-catenin signaling is known to up-regulate it (Arnold et al., 2000; Schohl and Fagotto, 2003), we speculate that both vertebrate and ascidian *Zic* genes have biphasic roles in notochord development: at an early stage, they play a role in the generation of the mesodermal or early notochord precursor cells, and later, they limit the extent of notochord precursor cell differentiation by inhibiting Wnt/β-catenin signaling.

Additional contact points between the *Zic* genes and Wnt/β-catenin signaling can be seen in chordate development. In ascidian embryo, *ZicL* is identified as a β-catenin downstream gene (Imai et al., 2002). If we consider the *Xenopus* embryos before starting gastrulation, upregulation of *Zic1*, *Zic2* and *Zic3* in the organizer region (Supplemental Figs. 1) can be a downstream event of Wnt/β-catenin signaling because this signaling is known to be essential for the establishment of organizer (Clevers, 2006). In addition, a recent study showed that macho-1, an ascidian Zic homologue, regulates the unequal cell division in cooperation with β-catenin via a mechanism independent of the transcriptional activity of macho-1 (Kumano et al., 2010). This reminds us of transcriptional activity-independent β-catenin suppression by Zic2, and analogous role would be worth being tested in vertebrate Zic proteins. Although still not conclusive, the interaction between Zic and Wnt/β-catenin signaling may be sequential and bidirectional in the mesoderm/notochord development.

Mechanism underlying Zic3-mediated suppression of Wnt-signaling

In this study, we obtained three clues to the mechanism underlying Zic3-mediated suppression of Wnt/β-catenin signaling: the necessity of Zic3 nuclear localization, the decreased nuclear localization and enhanced degradation of β-catenin by Zic3, and the physical interaction of Zic3 with Tcf1 and XIC. Furthermore, the suppressive effect of Zic3 on β-catenin mediated transcriptional activation was not reproducible in some mammalian cell lines (data not shown), suggesting the presence of tissue specific Zic3 nuclear target(s), X (Fig. 8). On the basis of these facts, we hypothesize that Zic3 may target X directly or indirectly in notochord precursor cell, and X can inhibit β-catenin-mediated transcriptional activation (Fig. 8). The decrement of nuclear β-catenin and enhanced degradation of β-catenin may reflect the ensuing events caused by the β-catenin suppression in cell nuclei. Although the XIC or Tcf can be the candidate factors for X, our attempts to prove the involvement of them in the Zic3-mediated suppression failed in the reporter gene (TOPFLASH) and immunoprecipitation assays *in vivo* (data not shown). Since the decreased nuclear localization and enhanced degradation of β-catenin were observed by the Zic3 overexpression, further exploration for X should include the factors that can influence the β-catenin dynamics. For example, recent studies revealed that TCF/LEF is degraded by a mechanism mediating context-dependent ubiquitin/proteasome system (Ishitani et al., 1999; Yamada et al., 2006), which might be related to the decrement of TCF in the immunoprecipitation assay (Fig. 7).

On the other hand, another feasible mechanism for Zic3-mediated inhibition of Wnt signaling is drawn from the role of XIC on the Siamois-mediated activation of the *Goosecoid* promoter (Snider and Tapscott, 2005). XIC is known to enhance the transcriptional activation process, and the knockdown of XIC results in the loss of dorsoanterior structures, including the notochord (Snider and Tapscott, 2005). If XIC is functionally sequestered by Zic3 expression, it could affect notochord development (Fig. 8). The partial rescue of Zic3 overexpression by Siamois co-expression might be explained by the

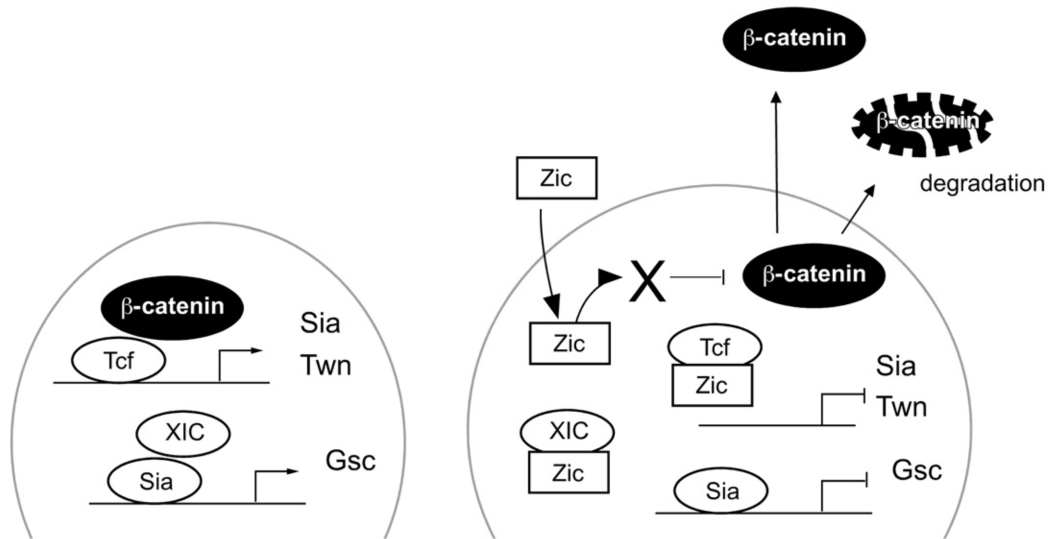

Fig. 8. Hypothetical mechanism underlying Zic3-mediated inhibition of Wnt/β-catenin signaling.

presumptive sequestration of XIC by Zic3, although, in this regard, the involvement of other Wnt/β-catenin signaling targets, such as Nr3, cannot be excluded.

Implications of the Zic3–Wnt signaling interaction

Zic3–Wnt interactions may have implications for several biological processes because Wnt signaling is widely involved in virtually every aspect of embryonic development as well as in homeostatic self-renewal in a number of adult tissues (Clevers, 2006). In particular, Wnt/β-catenin signaling pathway components are highly related to oncogenesis (Moon et al., 2002; Nusse, 2005b), and *Zic* expression is changed or detected in several types of tumors (Aruga et al., 2010; Bataller et al., 2004; Sabater et al., 2008; Wang et al., 2009).

Several aspects of the relationship between Zic proteins and Wnt/β-catenin signaling are worthy of further investigation. First, the other *Xenopus Zic* genes (*Zic1*, *Zic2*, *Zic4*, and *Zic5*) also suppressed the Wnt/β-catenin signaling pathway in a TOPFLASH reporter gene assay. Furthermore, their expression profiles partially overlap that of *Zic3* and their overexpression phenotypes are partially similar (Supplemental Figs. 1 and 2; Fujimi et al., 2006; Nakata et al., 1998; 2000). These results suggest that not only Zic3 but also the other four Zic proteins can be modulators of Wnt/β-catenin signaling *in vivo*.

Second, Zic3 can bind Tcf1 and Tcf3 (Fig. 7 and data not shown). Although the interaction may not fully account for the Wnt-signaling suppression in the *Xenopus* gastrula, the functional modulation of the Tcf/β-catenin molecular complex by Zic3 is possible in cell nuclei. In rodent embryonic stem cells, Zic3 colocalized with Tcf3 (data not shown), suggesting that the interaction can occur in mammalian pluripotent cells. Considering the necessity of Zic3 (Lim et al., 2007) and Tcf3 (Cole et al., 2008; Yi et al., 2008) in maintaining the integrity of embryonic stem cells, the interactions between them may contribute to the functions attributed to each.

Finally, both *Zic* genes (Aruga et al., 2006; Lindgens et al., 2004) and Wnt/β-catenin signaling systems (Petersen and Reddien, 2009) may be essential metazoan toolkit programs for development. Interactions at both the protein-to-protein level and in gene expression regulatory networks have been described (Benedyk et al., 1994; Inoue et al., 2007a; Merzdorf and Sive, 2006; Nagai et al., 1997). We suggest that the analysis of Zic–Wnt/β-catenin signaling interactions would also be an intriguing topic from evolutionary perspectives.

Conclusion

In this study, we demonstrated that *Xenopus* Zic3 had a role for development of notochord and Spemann's organizer, mediated by the Wnt/β-catenin signaling attenuation. Molecular mechanisms for the attenuation by Zic3 included, 1) necessity of Zic3 nuclear localization, 2) reduction of β-catenin nuclear accumulation and enhancement of β-catenin degradation, and 3) interactions with components modulating the signaling activity (Tcfs and XIC). The results provided evidence for a novel system for fine-tuning of Wnt/β-catenin signaling, and might be clues to expand understanding on the biological significances of Zic proteins.

Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.ydbio.2011.10.026.

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