PP2A:B56ε is required for Wnt/β-catenin signaling during embryonic development

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Summary

The Wnt/β-catenin pathway plays important roles during embryonic development and growth control. The B56 regulatory subunit of protein phosphatase 2A (PP2A) has been implicated as a regulator of this pathway. However, this has not been investigated by loss-of-function analyses. Here we report loss-of-function analysis of PP2A:B56ε during early *Xenopus* embryogenesis. We provide direct evidence that PP2A:B56ε is required for Wnt/β-catenin signaling upstream of Dishevelled and downstream of the Wnt ligand. We show that maternal PP2A:B56ε function is required for dorsal development, and PP2A:B56ε function is required later for the expression of the Wnt target gene *engrailed*, for subsequent midbrain-hindbrain boundary formation, and for closure of the neural tube. These data demonstrate a positive role for PP2A:B56ε in the Wnt pathway.

Supplemental data available online

Key words: Wnt/β-catenin, Protein phosphatase 2A, B56, *Xenopus*, Embryo

Introduction

The Wnt pathway is a highly conserved signaling pathway that plays essential roles in animal development and human disease (Moon et al., 2002; Wodarz and Nusse, 1998). Within the cell, the Wnt pathway has several branches, including the canonical Wnt pathway (Wnt/β-catenin pathway), the planar cell polarity (PCP) pathway and the Wnt/Ca^{2+} pathway. Both the Wnt/β-catenin pathway and the PCP pathway have been studied extensively in early embryonic development. Wnt/β-catenin signaling is involved in tumorigenesis, embryonic patterning, cell proliferation and cell fate determination (Moon et al., 2002; Wodarz and Nusse, 1998) in diverse organisms from Hydra to mammals. In contrast, the PCP pathway, which shares the Wnt/Wingless receptor (Frizzled) and Dishevelled (Dsh) with the Wnt/β-catenin pathway, regulates cell polarity in *Drosophila*, and cell movements during gastrulation in vertebrates (Mlodzik, 2002; Tada et al., 2002; Wallingford et al., 2000). However, the mechanism by which these pathways are precisely regulated during development remains largely unclear.

Activation of the canonical Wnt/β-catenin pathway leads to stabilization and accumulation of β-catenin, which in turn activates transcription of Wnt target genes. In the absence of Wnts, cytosolic β-catenin binds to the axin complex, where it is phosphorylated and targeted for rapid proteosomal degradation. Binding of Wnts to the coreceptors frizzled and arrow/LRP inhibits β-catenin phosphorylation and turnover, allowing β-catenin to accumulate and thus to activate TCF/Lef-mediated transcription. In *Drosophila*, this pathway requires Dsh, a cytoplasmic protein of unclear function, which has been placed downstream of the receptor and upstream of the degradation complex (Moon et al., 2002). This pathway plays several roles during vertebrate development. Wnt signaling prior to gastrulation is required for primary axis formation in vertebrates (De Robertis et al., 2000; Harland and Gerhart, 1997; Heasman, 1997; Liu et al., 1999). At a later stage, the canonical Wnt pathway inhibits development of anterior structures, including the head (De Robertis et al., 2000; Harland and Gerhart, 1997; Heasman, 1997; Mukhopadhyay et al., 2001). In addition, this pathway is required for midbrain-hindbrain formation (McGrew et al., 1999; McMahon et al., 1992) and neural crest development (reviewed by Wu et al., 2003).

Protein phosphatase 2A (PP2A) is a heterotrimeric complex consisting of a catalytic subunit (C), a structural subunit (A), and a variable regulatory subunit (B), including B55, B56 and PR72, which are believed to regulate the specificity and activity of PP2A (McCright and Virshup, 1995). PP2A has been proposed to be involved in the Wnt/β-catenin pathway, based on the observations that the C and B56 subunits physically interact with Wnt pathway components and that overexpression of PP2A subunits antagonizes Wnt/β-catenin signaling. In addition, treatment of HEK 293 cells with okadaic acid, an inhibitor of PP2A, results in elevated β-catenin protein levels (Seeling et al., 1999). The epsilon isoform of B56 (PP2A:B56ε) interacts with dsh and with the adenomatous polyposis coli (APC) protein in yeast two hybrid assays; furthermore, overexpression of *PP2A:B56ε* inhibits Wnt/β-catenin signaling in tissue culture and in *Xenopus* embryos (Gao et al., 2002; Li et al., 2001; Ratcliffe et al., 2000; Seeling et al., 1999), although the mechanism of this inhibition remains unclear. Analysis of the in vivo function of PP2A:B56 subunits

during embryonic development by loss-of-function approaches has been hampered in part by the apparent requirement for these subunits in cell survival; loss of PP2A:B56 family members in fly causes early embryonic lethality (Hannus et al., 2002), and depletion of *PP2A:B56*, by RNA interference (RNAi), in S2 cells induces apoptosis (Li et al., 2002). Recently, however, *widerborst*, a fly mutant for *PP2A:B56*, was identified in a screen for genes in the PCP pathway. The *widerborst* phenotype includes formation of multiple wing hairs and abnormal hair polarity in the wing blade. Further characterization placed *widerborst* upstream of *dsh* and *flamingo* (*fla*) in the PCP pathway. In zebrafish, knocking down *widerborst* function inhibited convergent extension (Hannus et al., 2002), a type of cell movement during vertebrate gastrulation that is probably regulated by the PCP pathway (Tree et al., 2002). Depletion of *widerborst* also altered the expression domain of *goosecoid*, a dorsally restricted organizer gene, an effect that could represent disruption of either canonical or noncanonical (PCP) Wnt signaling (Hannus et al., 2002). Therefore, a role for PP2A:B56 in canonical Wnt/β-catenin signaling has not yet been addressed directly by loss-of-function analyses in any species.

Materials and methods

Embryo manipulations

For routine embryological experiments, *Xenopus laevis* embryos were obtained and microinjected as described (Sive et al., 2000). Host transfer was performed according to a previously published protocol (Heasman et al., 1991). Briefly, oocytes were obtained by manual defolliculation and injected with various amounts of morpholino oligonucleotides and/or mRNA as described in the text. Injected oocytes were cultured in oocyte culture medium (Heasman et al., 1991) for 24 hours and then treated with 2 nM progestrone. Matured oocytes were stained with vital dyes and transferred into an anesthetized female frog that was laying eggs. Transferred eggs were recovered and fertilized.

Plasmid construction and morpholino

pCS2-PP2A:B56ε was constructed by cloning a PCR fragment containing the *PP2A:B56ε* (AF298157, a gift from M. J. Ratcliffe) opening reading frame into pCS2-FLAG. pCS2-PP2A:B56ε-c was generated by site-directed mutagenesis using the Quick-Change kit (Stratagene). All constructs were verified by sequencing.

Morpholinos were ordered from Gene Tools (Corvallis, OR). The sequence of the morpholino oligonucleotide directed against *PP2A:B56ε* was: 5′-GAGGAGTGGTTGGTGCTGAGGACAT-3′; The mismatched control morpholino had the sequence 5′-GAcGAcTGGTTGcTGCTGAcGAgAT-3′; and the standard control morpholino had the sequence 5′-CCTCTTACCTCAGTTACAA-TTTATA-3′.

RT-PCR and whole-mount in situ hybridization

RNA extraction and RT-PCR methods were as described previously (Yang et al., 2002). For host transfer experiments, two embryos were included in each sample. For routine experiments, five embryos were included in each sample. Whole-mount in situ hybridization was performed as described previously (Deardorff et al., 1998).

Western blots

For western blot analysis, embryos or oocytes were homogenized (10 μl per embryo) in modified RIPA buffer (159 mM NaCl, 50 mM Tris, pH 8, 1% NP40, 0.5% deoxycholate, 2 mM EDTA, 20 μg/ml aprotinin, 40 μg/ml leupeptin, 4 μg/ml pepstatin, 0.75 mM PMSF, 25 mM β-glycerophosphate, 1 mM Na_3Vo_4, 100 mM NaF). Lysates were centrifuged three times at 14,000 ***g*** for 10 minutes in a tabletop centrifuge at 4°C. Supernatant was collected. For cytoplasmic β-catenin analysis (Fagotto et al., 1999), 50 μl lysate was incubated with 50 μl ConA-agarose beads (Calbiochem) at 4°C with rotation. Beads were removed 2 hours later by centrifuging at 500 ***g*** for 5 minutes at 4°C. Protein loading buffer was included in each sample. Each sample was boiled at 100°C for 5 minutes and separated by 7.5% SDS-PAGE electrophoresis. Western blots were performed according to standard protocol with anti-*Xenopus* β-catenin (rabbit serum, 1:1000), anti-EGFP (Clontech, 1:1000), anti-Myc (9E10, Santa Cruz, 1:1000), anti-FLAG (M2, Sigma, 1:1000) and anti-hnRNP (1:2000; gift from Gideon Dreffuss, University of Pennsylvania).

Results

Expression of *PP2A:B56ε* during *Xenopus* embryonic development

PP2A:B56ε, a member of the B56 family of PP2A regulatory subunits (McCright and Virshup, 1995), has been implicated in Wnt signaling in vertebrate embryos and in cultured cells (Gao et al., 2002; Li et al., 2001; Ratcliffe et al., 2000; Seeling et al., 1999), but the expression pattern during embryonic development has not been reported. To further identify a possible role for PP2A:B56ε in Wnt/β-catenin signaling during embryogenesis, we investigated the spatial and temporal pattern of *PP2A:B56ε* expression pattern during early *Xenopus* development.

PP2A:B56ε is maternally expressed (not shown) and uniformly distributed in the embryo prior to midblastula transition (MBT) (Fig. 1A,B). At the late blastula stage, zygotic expression of *PP2A:B56ε* begins in the dorsal marginal zone (Fig. 1C), partially overlapping the expression domain of two organizer-specific genes, *goosecoid* (*gsc*) (Cho et al., 1991) and *cerberus* (Bouwmeester et al., 1996) (data not shown). The expression of *PP2A:B56ε* expands to the lateral and ventral marginal zone shortly after the onset of gastrulation (Fig. 1D). During the early neurula stage, it is expressed exclusively in the neural ectoderm throughout its anterior-posterior extent (Fig. 1E,F). Little if any expression can be detected in the underlying mesoderm. The expression of *PP2A:B56ε* persists in the anterior region until the tadpole stage, when strong expression is detected in the eyes and branchial arches (Fig. 1G). The dynamic expression pattern of *PP2A:B56ε* suggests that it may play important roles during early embryonic development.

Maternal PP2A:B56ε activity is required for dorsal development

To investigate the function of *PP2A:B56ε* during early embryonic development, we designed a morpholino antisense oligonucleotide to block its translation (Heasman et al., 2000). The morpholino directed against *PP2A:B56ε* inhibited the translation of flag-tagged wild type *PP2A:B56ε* mRNA in *Xenopus* oocytes (Fig. 2A), but not the translation of a modified mRNA (*PP2A:B56ε-c*) lacking the morpholino target sequence (also flag-tagged). In addition, an unrelated control morpholino had no effect on the translation of *PP2A:B56ε* (data not shown).

Because *PP2A:B56ε* is expressed maternally, we took advantage of the host transfer technique, in which oocytes are injected with antisense oligonucleotides, matured ex vivo, and

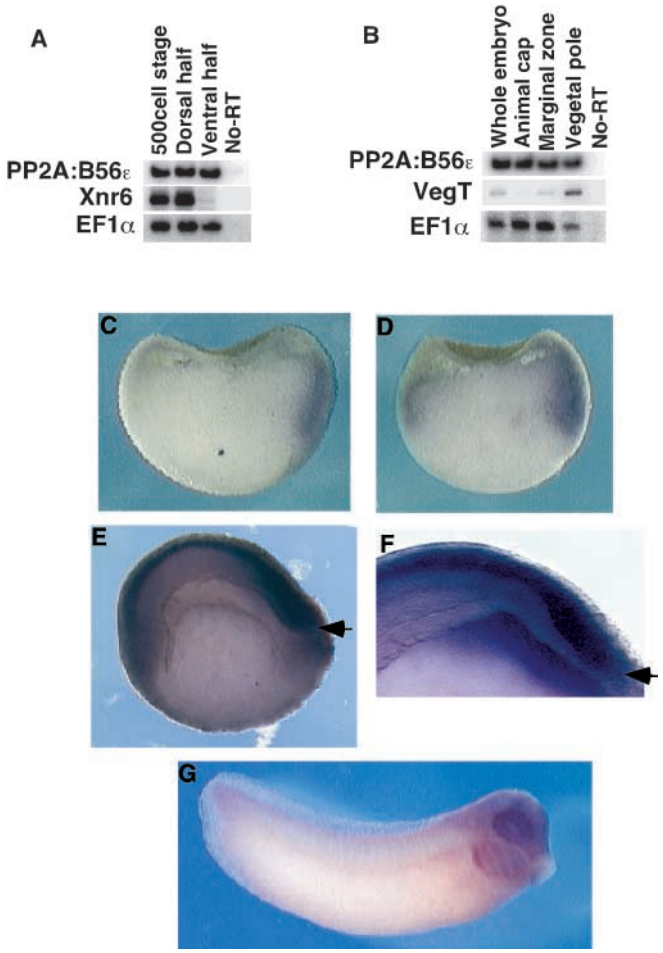

Fig. 1. Expression pattern of *PP2A:B56ε* during early embryonic development of *Xenopus*. (A,B) Expression of *PP2A:B56ε* in the early blastula. (A) *PP2A:B56ε* expression along the dorsal-ventral axis: 500-cell stage embryos were dissected into dorsal and ventral halves. RNA was prepared from each half. Expression of *PP2A:B56ε* was analyzed by RT-PCR. *EF1α* was used as loading control. *Xnr6* (dorsally restricted expression) was used as a control for accurate dissection. (B) *PP2A:B56ε* expression along the animal-vegetal axis: 500-cell stage embryos were dissected into animal, marginal zone, and vegetal pieces. RNA was prepared from each and expression of *PP2A:B56ε* was analyzed by RT-PCR. *VegT* (vegetal pole restricted expression) was used as a control for accurate dissection. (C-F) *PP2A:B56ε* expression as determined by in situ hybridization of hemisections. (C) *PP2A:B56ε* expression in the dorsal marginal zone of an early gastrula stage (stage 10) embryo. (D. Expression in marginal zone of a midgastrula (stage 11) embryo; (E) expression in neural ectoderm of a midneurula stage (stage 15) embryo. (F) Higher magnification of E showing *PP2A:B56ε* expression in the anterior neural ectoderm. (G) *PP2A:B56ε* expression in a stage 33 tadpole. Arrows in E and F indicate the anterior edge of *PP2A:B56ε* expression domain in the neural ectoderm.

reimplanted into a foster mother to complete ovulation (Heasman et al., 1991). After egg laying, eggs are fertilized and development of transferred embryos is followed morphologically and by analysis of cell-type-specific molecular markers.

Depletion of maternal *PP2A:B56ε* resulted in a severe defect in dorsal development. Host-transfer embryos previously injected with the *PP2A:B56ε* morpholino did not form a dorsal blastopore lip (Fig. 2C), a morphological marker of the Spemann organizer (Harland and Gerhart, 1997), while sibling controls formed a normal dorsal lip at stage 10 (Fig. 2B). Later development could not be assessed in *PP2A:B56ε* morpholino-injected embryos derived from host transfer, as the embryos died in early neurula stages. This embryonic lethality does not appear to be a nonspecific effect of morpholino injection or the host transfer technique, as oocytes injected with 40 ng of control morpholino or 10 ng of a control mismatched morpholino (similar in sequence to the *PP2A:B56ε* morpholino, but containing 5 point mutations) developed relatively normally when carried through the host-transferred procedure.

To confirm that depletion of maternal *PP2A:B56ε* interfered with dorsal development, host-transfer embryos were harvested at the midgastrula stage and expression of dorsal-specific genes was analyzed by RT-PCR (Fig. 2D). The expression of the dorsal-specific genes *noggin* (Smith and Harland, 1992) *gsc*, *cerberus*, *dkk1* (Glinka et al., 1998), *siamois* (Lemaire et al., 1995) and *Xnr3* (Smith et al., 1995) was markedly reduced in PP2A:B56ε knockdown embryos while the expression of the ventrally restricted genes *sizzled* (Salic et al., 1997) and *Xvent2* (Onichtchouk et al., 1996) was increased, consistent with the loss of the dorsal blastopore lip. Since the expression of all dorsal-specific genes analyzed in these experiments was reduced, we conclude that maternal PP2A:B56ε function is required for dorsal gene expression and organizer formation in *Xenopus* embryos.

This loss of dorsal development and dorsal gene expression in embryos lacking maternal PP2A:B56ε is highly similar to depletion of maternal β-catenin, which is required for dorsal axis specification (Heasman et al., 1994; Heasman et al., 2000; Xanthos et al., 2002). Therefore, we directly compared gene expression profiles in gastrulae derived from embryos in which maternal PP2A:B56ε or β-catenin had been depleted. Consistent with previous observations (Heasman et al., 1994; Heasman et al., 2000; Xanthos et al., 2002), interfering with maternal β-catenin function resulted in down-regulation of all dorsal markers analyzed at this stage, including *noggin*, *gsc*, *cerberus*, *dkk1*, *siamois* and *Xnr3*, similar to the PP2A:B56ε knockdown embryos. Furthermore, the expression of *sizzled*, and *Xvent2*, two ventral genes, was increased in both PP2A:B56ε and β-catenin knockdown embryos, while *Xvent1*, *sox17*, *mixer*, *msx1*, and zygotic *PP2A:B56ε* did not change significantly in either group (Fig. 2D).

This loss of dorsal development after knockdown of maternal PP2A:B56ε and the striking similarity to embryos lacking maternal β-catenin raises the intriguing possibility that PP2A:B56ε is required for maternal Wnt/β-catenin signaling. To explore this possibility further, we examined maternal PP2A:B56ε knockdown embryos at earlier stages of development. The *Xenopus* nodal-related genes *Xnr5* and *Xnr6* require maternal β-catenin for expression (Takahashi et al., 2000), and both genes are transcribed prior to the midblastula transition (MBT) in a β-catenin-dependent manner (Yang et al., 2002). Expression of *Xnr5* and *Xnr6* was reduced in PP2A:B56ε knockdown embryos at the 1,000 cell stage (two cell divisions prior to MBT) (Fig. 2E). In addition, post-MBT expression of *Xnr3* and *siamois*, which also requires maternal

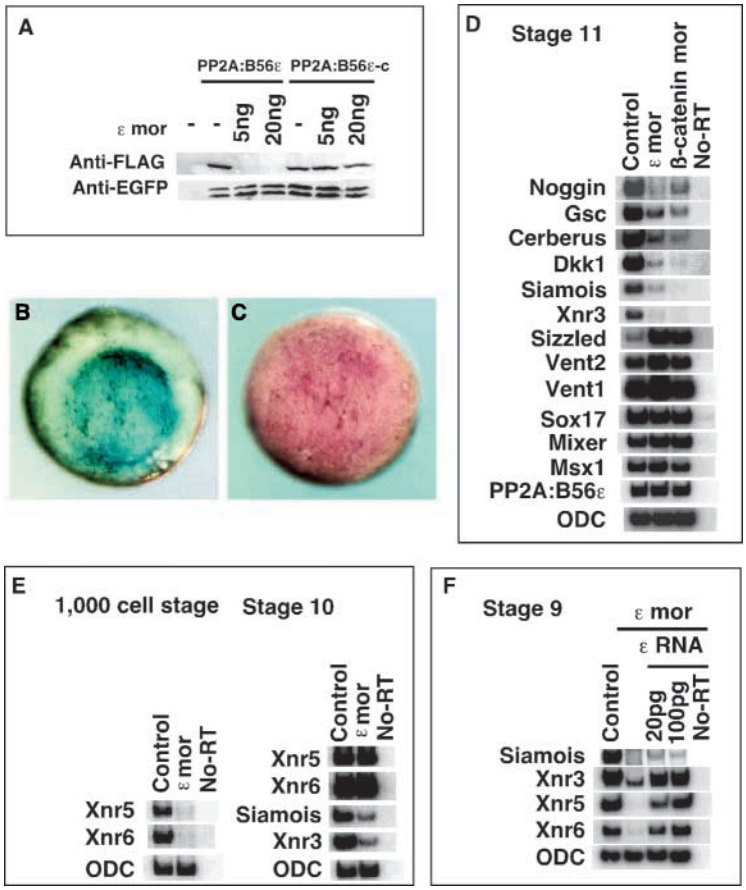

Fig. 2. Maternal PP2A:B56ε is required for dorsal development. (A) Morpholino antisense oligonucleotide directed against *PP2A:B56ε* blocks the translation of flag-tagged *PP2A:B56ε* RNA, but not flag-tagged *PP2A:B56ε*-c, in *Xenopus* oocytes, as determined by western blotting. EGFP mRNA was coinjected and western blotted with anti-GFP as a control. (B) Vegetal view of an uninjected host-transfer embryo at the onset of gastrulation (stage 10+), showing formation of the dorsal lip of the blastopore. (C) Vegetal view of a maternal PP2A:B56ε-depleted embryo also at stage 10+, showing absence of dorsal lip. (D) RT-PCR showing the gene expression profile in maternal PP2A:B56ε and β-catenin-depleted embryos at midgastrula stage (stage 11). (E) RT-PCR showing reduced expression of *Xnr5* and *Xnr6* at the 1,000-cell stage and reduced expression of *siamois* and *Xnr3* at gastrula stage (stage 10). (F) RT-PCR showing reduction of dorsal gene expression in maternal PP2A:B56ε-depleted embryos could be rescued by PP2A:B56ε-c mRNA.

β-catenin, was severely reduced at the early gastrula stages (stage 10) in PP2A:B56ε knockdown embryos (Fig. 2E). Thus, the early dorsal expression of multiple genes known to be targets of Wnt/β-catenin signaling requires maternal PP2A:B56ε.

To test whether the repression of dorsal gene expression by morpholino injection is specific to PP2A:B56ε loss-of-function, we rescued dorsal gene expression by coinjection of oocytes with the *PP2A:B56ε* morpholino and an mRNA (*PP2A:B56ε-c*) lacking the morpholino target sequence. Transferred embryos were harvested at stage 9 for gene expression analysis. *PP2A:B56ε-c* mRNA completely rescued the expression of *Xnr3*, *Xnr5* and *Xnr6* in a dose-dependent manner, and weakly rescued *siamois* expression at this stage (Fig. 2F). In addition, dorsal gene expression in host-transferred embryos injected with 10 ng of mismatched morpholino was relatively normal (data not shown). This suggests that the reduction of dorsal gene expression in PP2A:B56ε maternal depleted embryos is specific to PP2A:B56ε loss-of-function.

PP2A:B56ε activity is required for β-catenin stability

The effect of PP2A:B56ε depletion on the expression of targets of β-catenin signaling suggests that PP2A:B56ε may be required for Wnt/β-catenin signaling. To address this directly, we compared the accumulation of endogenous β-catenin protein, a well-established measure of canonical Wnt signaling, in control and maternal PP2A:B56ε-depleted embryos at the late blastula stage (stage 9), when Wnt/β-catenin signaling is maximally activated in *Xenopus* embryos. Consistent with the reduction in expression of β-catenin-regulated genes, cytosolic β-catenin protein was significantly decreased in maternal PP2A:B56ε–depleted embryos (Fig. 3A). In addition, depletion of PP2A:B56ε did not affect β-catenin protein levels in settings where Wnt signaling is not activated, such as oocytes or ventral blastomeres of maternally depleted embryos (data not shown). Therefore, we conclude that maternal PP2A:B56ε is required for Wnt-dependent accumulation of β-catenin protein.

PP2A:B56ε is required downstream of Wnt and upstream of dishevelled

Overexpression of positive regulators of the Wnt/β-catenin pathway, including *Wnt8b* (Cui et al., 1995), *Xdsh* (Sokol et al., 1995), *GSK-3 binding protein* (*GBP*) (Yost et al., 1998) or the GSK-3 interaction domain (*GID*) of axin (Hedgepeth et al., 1999) (both of which interact with and inhibit GSK-3 directly), β-*catenin* (Moon et al., 2002), and constitutively active *TCF* (Vonica et al., 2000), activates dorsal gene expression in *Xenopus* embryos. In order to define at which step PP2A:B56ε functions in Wnt/β-catenin signaling, we carried out epistatic analysis in the PP2A:B56ε loss-of-function background. Maternal PP2A:B56ε-depleted embryos were injected with mRNAs encoding constitutively active *TCF3* (*VP16-Xtcf3*), β-*catenin*, *GID*, *GBP*, *Xdsh* or *Wnt8b* into one dorsal blastomere at the 4-cell stage. The expression of *Xnr3* and *siamois* was analyzed by RT-PCR when embryos reached the gastrula stage. The reduced expression of *siamois* and *Xnr3* in maternal PP2A:B56ε-depleted embryos was rescued by *VP16-Xtcf3*, β-*catenin*, *GID*, *GBP* (not shown) and *Xdsh*. In contrast, *Wnt8b* (20 pg) failed to rescue the expression of *siamois* or *Xnr3* in maternal PP2A:B56ε-depleted embryos (Fig. 3B). These observations indicate that PP2A:B56ε is required for canonical Wnt signaling downstream of the Wnt ligand, but upstream of dsh.

PP2A:B56ε is required for midbrain-hindbrain boundary formation

The above observations suggest that maternal PP2A:B56ε

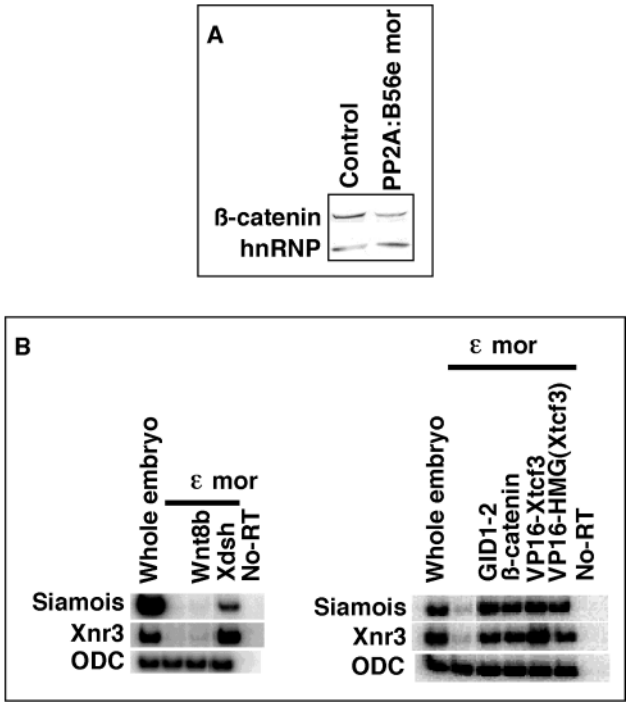

Fig. 3. PP2A:B56ε is required for Wnt/β-catenin signaling. (A) Western blot showing endogenous cytoplasmic β-catenin in maternal PP2A:B56ε-depleted embryos at stage 9. hn-RNP was used as a loading control. (Shown here is a representative result from three different experiments). (B) RT-PCR showing that injection of mRNA encoding *VP16-Xtcf3* (2 pg), β-*catenin* (500 pg), *GID* (1 ng), and *Xdsh* (2 ng) rescue *siamois* and *Xnr3* expression in maternal PP2A:B56ε-depleted embryos at stage 10, while *Wnt8b* (20 pg) does not. *ODC* was used as loading control. *Wnt8b* (20 pg), *Xdsh* (2 ng), *GID* (1 ng), β-catenin (500 pg), and *VP16-Xtcf3* (2 pg) induce similar percentages of secondary axis when injected ventrally into normal embryos at the 4-cell stage (not shown).

is required for Wnt/β-catenin signaling during early development. To investigate whether PP2A:B56ε is required for Wnt/β-catenin signaling at later stages, we explored the function of PP2A:B56ε in the neural ectoderm. It has been shown extensively that Wnt/β-catenin regulates formation of the midbrain-hindbrain boundary (MHB) in vertebrates (Joyner, 1996; McMahon et al., 1992). *engrailed 2* (*en2*), which marks this boundary during early stages of neural development (Hemmati-Brivanlou et al., 1991; Hemmati-Brivanlou and Harland, 1989), is a well-characterized target of the Wnt/β-catenin pathway (Joyner, 1996; McGrew et al., 1999). We therefore analyzed whether PP2A:B56ε function is required for *en2* expression and subsequent MHB formation. The PP2A:B56ε morpholino was injected at the 8-cell stage into the dorsal animal blastomeres that give rise to neural ectoderm (Moody, 1987), and expression of *en2*, as well as other neural markers, was analyzed by whole mount in situ hybridization and RT-PCR.

Expression of the MHB markers *en2* (Fig. 4A) and *wnt1* (Christian et al., 1991) (Fig. 4A) was severely reduced in

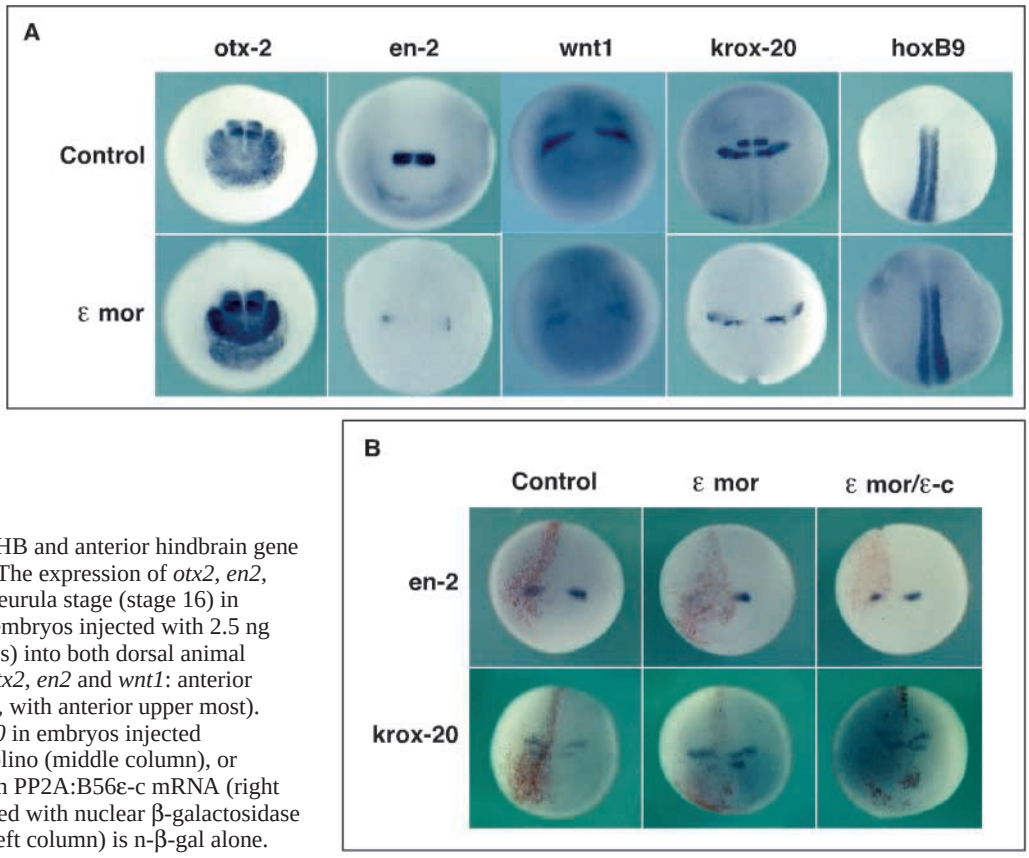

Fig. 4. PP2A:B56ε is required for MHB and anterior hindbrain gene expression in stage 16 neurulae. (A) The expression of *otx2*, *en2*, *wnt1*, *krox20* and *hoxB9* at the mid-neurula stage (stage 16) in control embryos (upper panels) and embryos injected with 2.5 ng PP2A:B56ε morpholino (lower panels) into both dorsal animal blastomeres at the 8-cell stage. For *otx2*, *en2* and *wnt1*: anterior view; *krox20* and *hoxB9*: dorsal view, with anterior upper most). (B) The expression of *en2* and *krox20* in embryos injected unilaterally with PP2A:B56ε morpholino (middle column), or PP2A:B56ε morpholino together with PP2A:B56ε-c mRNA (right column). All embryos were co-injected with nuclear β-galactosidase mRNA as a lineage tracer. Control (left column) is n-β-gal alone.

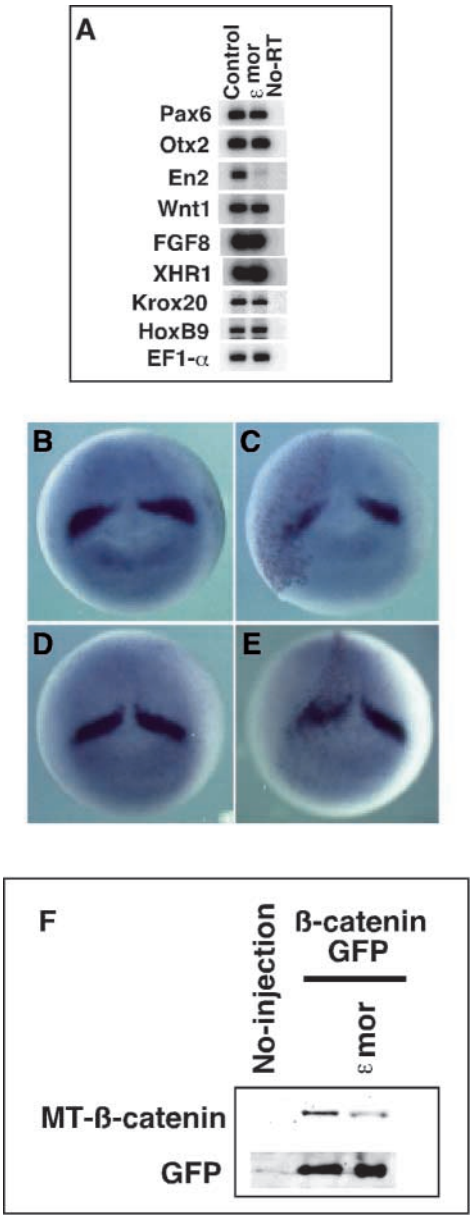

Fig. 5. Regulation of *en2* expression and β-catenin stability by PP2A:B56ε in the neural ectoderm at stage 14. (A) RT-PCR showing that *en2* is reduced by PP2A:B56ε morpholino at stage 14. (B-E) Whole-mount in situ hybridization showing *pax2.1* (B,C) and *XHR1* (D,E) are not reduced by PP2A:B56ε morpholino injection at stage 14. (B,D) Control embryos; (C,E) embryos injected with PP2A:B56ε morpholino unilaterally. (F) Western blot showing injected β-catenin was less stable in the neural ectoderm of PP2A:B56ε knockdown embryos. EFGP was used as a control for both injection and loading.

morpholino-injected embryos at the midneurula stage (stage 16). In addition, the anterior stripe of *krox20* (Bradley et al., 1993), which marks rhombomere 3, was also down-regulated. In contrast, the posterior stripe of *krox20* was only weakly reduced (Fig. 4A). *Otx2*, a forebrain marker (Lamb, 1993) and *hoxB9* (Fig. 4A), a spinal cord marker (Sharpe et al., 1987), were expressed normally. These changes in neural marker expression appear to be a specific consequence of PP2A:B56 loss-of-function, as co-injection of *PP2A:B56-c* mRNA rescued both *en2* and *krox20* (Fig. 4B) expression.

Interestingly, at an earlier stage of neural development (stage 14), the presumptive MHB region in *PP2A:B56ε* morpholino-injected embryos is present, with normal expression of *wnt1*, *fgf8* (Christen and Slack, 1997), *XHR1* (Shinga et al., 2001) and *Pax2.1* (Heller and Brandli, 1997). Of the markers tested, only *En2* was reduced by *PP2A:B56ε* morpholino at this stage (Fig. 5). (Forebrain markers *otx2* and *pax6*, the hindbrain marker *krox20*, and the spinal cord marker *hoxB9* were also normal; Fig. 5A.) Whole-mount in situ hybridization for *XHR1* and *pax2.1*, which are expressed exclusively in the MHB at this stage, was performed with embryos unilaterally injected with morpholino at the 8- to 16-cell stage and fixed at stage 14. Expression of *XHR1* (Fig. 5B) and *pax2.1* (Fig. 5D) on the injected side was indistinguishable from that on the uninjected side of the embryo, indicating that reduced *En2* expression in PP2A:B56ε knockdown embryos is not due to the loss of the presumptive MHB lineage at this earlier stage. These results suggest that PP2A:B56ε is required for expression of *en2*, a known target of Wnt signaling, and subsequently for the maintenance of the midbrain-hindbrain boundary.

The above observations raise the possibility that PP2A:B56ε is required for Wnt signaling during neural development. Thus, we compared the stability of β-catenin in the neural ectoderm of control and morpholino-injected embryos. mRNA encoding Myc-tagged β-catenin was injected alone or with morpholino against *PP2A:B56ε* into one dorsal animal blastomere at the 8-cell stage. Injected embryos were harvested at stage 14 and subjected to western blot analysis. Indeed, β-catenin accumulated in control neural ectoderm to a greater extent than that in PP2A:B56ε knockdown embryos. EGFP was used in this experiment as a lineage tracer to confirm that injected cells contributed to neural ectoderm (data not shown) and as a protein loading control for the western blot (Fig. 5F). Taken together, these results support the idea that PP2A:B56ε is required for Wnt/β-catenin signaling in the early neurula.

PP2A:B56ε is required for neural tube closure and proper head formation

In addition to the loss of *en2* expression and subsequent defects in later midbrain-hindbrain formation, PP2A:B56ε knockdown embryos exhibited dramatic defects in neural tube closure (Fig. 6B compare with 6A). The failure of neural tube closure in the anterior region occurred in all embryos injected with 2.5 ng morpholino, while defective posterior neural tube closure was observed at a lower frequency (varying from 10% to 30% in different clutches of eggs). The neural tube in most embryos injected with 2.5 ng of morpholino eventually closed 2 to 3 hours later than control embryos. (At a higher dose of morpholino (5 ng), the anterior neural tube failed to close, leading to lethality at the tadpole stage; data not shown.) Morpholino injection also delayed cement gland formation. At mid-neurula stage, cement gland formation could be seen as condensed pigmentation below the anterior neural ectoderm in controls (Fig. 6D); in morpholino-injected embryos, this pigmentation was reduced in intensity and was present in a broader region below the edge of the open anterior neural tube (Fig. 6E). Interestingly, pigmentation was completely absent in embryos with more severe neural tube defects (data not shown). At the swimming tadpole stage, morpholino-injected

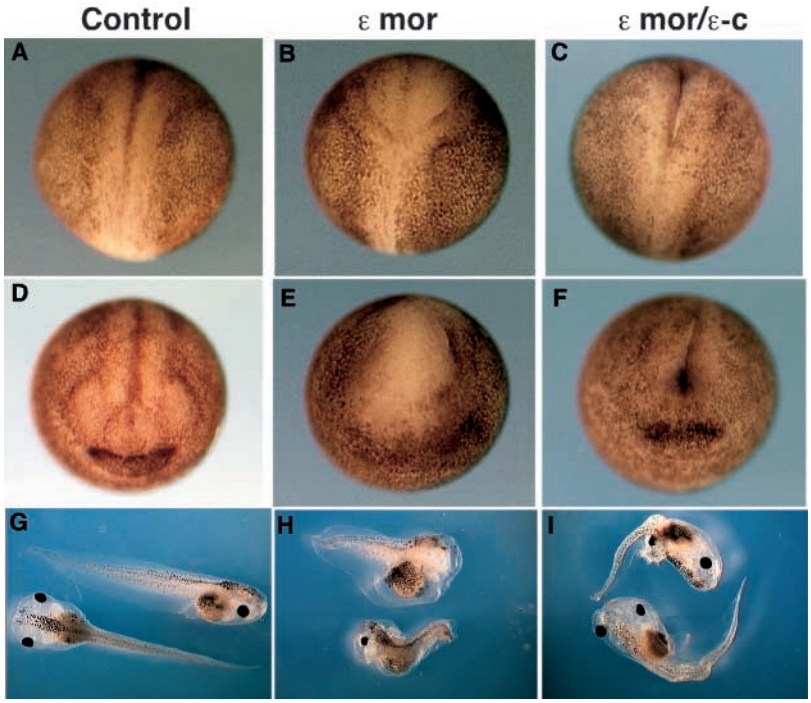

Fig. 6. PP2A:B56ε is required for neural tube closure and formation of head structures. (A-C) Dorsal views of stage 16 embryos. (A) Control; (B) embryo injected with 2.5 ng of PP2A:B56ε morpholino bilaterally; (C) embryo injected with 2.5 ng of PP2A:B56ε morpholino and 20 pg of PP2A:B56ε-c mRNA bilaterally. (D-F) Anterior view of stage 16 embryos. (D) Control embryo; (E) embryo injected with 2.5 ng of PP2A:B56ε morpholino bilaterally; (F) embryo injected with 2.5 ng of PP2A:B56ε morpholino and 20 pg of PP2A:B56ε-c mRNA bilaterally. (G-I) Tadpole stage. (G) Control; (H) embryo was injected with 2.5 ng of PP2A:B56ε morpholino; (C) embryo was injected with 2.5 ng of PP2A:B56ε morpholino and 20 pg of PP2A:B56ε-c mRNA.

embryos had smaller heads and either cyclopic eyes or no eyes (Fig. 6H). These phenotypes appeared to be due to the lack of PP2A:B56ε in the neural ectoderm, as 20 pg of *PP2A:B56ε-c* mRNA rescued these phenotypes (Fig. 6C,F,I), including the lethal phenotype caused by 5 ng of morpholino injection (data not shown). Embryos injected with the same dose of mismatched morpholino developed relatively normally (data not shown). Therefore, we conclude that PP2A:B56ε is required for neural tube closure and proper formation of head structures.

Discussion

In this report, we show that *PP2A:B56ε* is dynamically expressed during *Xenopus* embryonic development. PP2A:B56ε appears to be required for dorsal axis specification, neural tube closure, MHB formation, and proper cement gland and eye development as well. Furthermore, PP2A:B56ε is required for Wnt-mediated β-catenin stabilization upstream of dsh and downstream of the Wnt ligand. Hence, our data suggest a positive role for PP2A:B56ε in Wnt/β-catenin signaling.

PP2A:B56ε is required for Wnt/β-catenin signaling

Loss-of-function analysis in *Drosophila* and zebrafish suggested that PP2A:B56 family members play roles in the PCP pathway upstream of dsh (Hannus et al., 2002). Although Frizzled and Dsh function in both the PCP pathway and the canonical Wnt/β-catenin pathway (Mlodzik, 2002), the requirement for PP2A:B56ε upstream of Dsh in Wnt/β-catenin signaling has not previously been shown.

The loss-of-function analysis of PP2A:B56ε presented here directly supports the hypothesis that PP2A:B56ε is required for Wnt/β-catenin signaling. PP2A:B56ε is required for the expression of multiple Wnt/β-catenin target genes, including *Xnr5*, *Xnr6* during pre-MBT stages, *Xnr3*, *siamois*, *noggin*, *goosecoid*, *cerberus* and *Xdkk1*, during the gastrula stage, and *en2* during neurulation. Furthermore, PP2A:B56ε is required for β-catenin accumulation in cells that respond to endogenous Wnt signaling, such as dorsal blastomeres in blastula-stage embryos and in the neural ectoderm of early neurulae. In contrast, the constitutive turnover of β-catenin in ventral blastomeres and in oocytes is not sensitive to PP2A:B56ε depletion (not shown). The requirement for PP2A:B56ε in canonical Wnt signaling is further supported by epistatic analysis. Dorsal gene expression is rescued by dsh and downstream Wnt signaling components but not by Wnt8b. Based on these observations, we conclude that PP2A:B56ε is required for Wnt/β-catenin signaling downstream of the Wnt ligand and upstream of dsh.

In contrast to our observations with depletion of PP2A:B56ε, overexpression of PP2A:B56 family members inhibits Wnt/β-catenin signaling (Gao et al., 2002; Li et al., 2001; Ratcliffe et al., 2000; Seeling et al., 1999). Other B56 family members, such as B56α, may have different functions in the Wnt/β-catenin pathway. For example, we found that overexpression of *PP2A:B56ε* in dorsal blastomeres leads to anterior truncation of embryos without altering dorsal gene expression (see Fig. S1 at http://dev.biologists.org/supplemental/), a phenotype that is distinct from the strong ventralization caused by *PP2A:B56α* overexpression (Li, 2001). These observations are consistent with the suggestion that the two isoforms have different functions in the Wnt pathway. However, overexpressed PP2A:B56ε also inhibits secondary dorsal axis induction by positive modulators of Wnt/β-catenin pathway (Ratcliffe et al., 2000) (see Table S1 at http://dev.biologists.org/supplemental/). To explain this, PP2A:B56ε could regulate multiple steps in Wnt signaling, and loss-of-function at an upstream step may obscure an inhibitory role at a downstream step in the pathway. Alternatively, overexpression of PP2A:B56 or other subunits that interact with the PP2A complex could sequester essential components of the phosphatase complex and interfere with its normal function. It also remains unclear whether PP2A:B56ε regulates Wnt signaling through modulation of PP2A activity. Further experiments are needed in order to understand the mechanism by which PP2A:B56ε regulates the Wnt/β-catenin pathway.

Similar to observations that B56 family members are required for convergence and extension movements in zebrafish gastrulation (Hannus et al., 2002), we find that PP2A:B56ε is

required for neural tube closure in *Xenopus*. The PCP pathway has been proposed to regulate the cell movements involved in neural tube closure in *Xenopus* and mouse (Hamblet et al., 2002; Wallingford and Harland, 2001; Wallingford and Harland, 2002). Since PP2A:B56ε is required for Wnt signaling upstream of dsh, it would be of interest to test whether PP2A:B56ε is a common component in both non-canonical and canonical Wnt/frizzled signaling upstream of dsh.

PP2A:B56ε is required for early embryonic development

Consistent with the *PP2A:B56ε* expression pattern during early embryonic development, our loss-of-function analysis suggests that maternal PP2A:B56ε is required for dorsal-ventral patterning. Maternal depletion of PP2A:B56ε inhibits dorsal lip formation, a morphological marker for the Spemann organizer. The expression of multiple dorsal-specific genes is reduced in maternal PP2A:B56ε-depleted embryos while ventral gene expression is increased. This gene expression profile in maternal PP2A:B56ε-depleted embryos is reminiscent of embryos lacking maternal β-catenin (Heasman et al., 1994; Heasman et al., 2000; Xanthos et al., 2002). Our data, together with previous work showing that maternal *Xenopus frizzled7* is required for dorsal development (Sumanas et al., 2000), suggest that components of the Wnt/β-catenin pathway, upstream of dsh are involved in primary dorsal axis specification during *Xenopus* embryonic development.

The inhibition of dorsal development as well as dorsal gene expression appears to be specific to PP2A:B56ε loss-of-function, as PP2A:B56ε-c mRNA, which lacks the morpholino target sequence, rescues the expression of most dorsal genes. Interestingly, *siamois* expression was not rescued efficiently by PP2A:B56ε-c. In addition, the *PP2A:B56ε* morpholino causes an embryonic lethal phenotype, similar to homozygous *widerborst* mutants (Hannus et al., 2002), which was not seen in embryos injected with control morpholinos or the β-catenin morpholino. Although dorsal gene expression and dorsal lip formation were rescued with PP2A:B56ε-c mRNA, we were unable to rescue the embryonic lethality, and thus we cannot rule out the possibility that the morpholino causes some additional non-specific defects at a later stage in host transfer experiments.

In addition to the role of maternal PP2A:B56ε in dorsal-ventral patterning, PP2A:B56ε is also required in neural ectoderm for expression of the Wnt target gene *en2*. Depletion of PP2A:B56ε blocks the expression of *en2* in the early neurula (stage 14), but not other early MHB markers, including *fgf8*, *wnt1*, *pax2.1* and *XHR1*, suggesting that PP2A:B56ε is specifically required for *en2* expression but not for the specification of the MHB lineage. Interestingly, the phenotype of morpholino-injected embryos becomes more severe at the mid-neurula stage (stage 16), with reduction of MHB markers (*wnt1* and *en2*) and an anterior hindbrain marker (the anterior stripe of *krox20*). In contrast, the anterior marker *otx2* (Lamb et al., 1993), the spinal cord marker *hoxB9* and the posterior stripe of *krox20* appear to be normal in PP2A:B56ε morpholino-injected embryos. This phenotype is reminiscent of the remarkably restricted phenotype observed in the mouse *wnt1* knockout (McMahon et al., 1992) and the β-catenin conditional knockout in *wnt1*-expressing cells (Brault et al., 2001), and further supports the requirement of Wnt/β-catenin for *en2* expression and for the maintenance of the MHB. The PP2A:B56ε morpholino did not reduce *slug* expression, a target of Wnt/β-catenin signaling in the neural crest lineage (not shown), suggesting that other B56 family members may function redundantly during embryonic development.

Interfering with PP2A:B56ε also causes phenotypes that are not clearly related to impaired Wnt/β-catenin signaling. For example, depletion of PP2A:B56ε in dorsal-animal blastomeres leads to delayed neural tube closure at the neurula stage, and causes lethality at the tadpole stage, which is not seen with depletion of β-catenin. In addition, cement gland formation in PP2A:B56ε knockdown embryos is delayed, in contrast to the enlarged cement gland phenotype observed with downstream Wnt antagonists (Itoh et al., 1995; Kofron et al., 2001). These observations are consistent with additional roles for PP2A:B56ε independent of the Wnt pathway.

In summary, our data directly support the requirement of PP2A:B56ε for Wnt/β-catenin signaling. PP2A:B56ε is required in several developmental processes regulated by Wnt/β-catenin signaling, including primary dorsal axis specification, formation of the midbrain-hindbrain boundary, and closure of the neural tube. PP2A:B56ε may thus function during early embryonic development through the canonical Wnt pathway dependent, as well as through Wnt-independent pathways.

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Table S1. PP2A:B56ε blocks secondary axis induced by β-catenin and Xtcf3-VP16, but not by siamois and Xtwin

mRNA injected	Axis duplication(%)	Normal axis(%)	*n*
β-catenin (250pg)	69	31	74
β-catenin (250pg)+PP2A:B56ε (100pg)	4	96	50
Xtcf3-VP16 (0.5pg)	93	7	68
Xtcf3-Vp16 (0.5pg)+PP2A:B56ε (100pg)	6	94	63
Siamois (10pg)	48	52	85
Siamois (10pg)+PP2A:B56ε (100pg)	49	40	78
Xtwin (5pg)	97	3	71
Xtwin (5pg)+PP2A:B56ε (100pg)	96	4	85

β-catenin, Xtcf3-VP16, siamois and Xtwin induce secondary axis if the respective mRNAs are injected ventrally at the four-cell stage. PP2A:B56ε (100pg) inhibits secondary axis induced by β-catenin and Xtcf3-VP16, but not by siamois and Xtwin [as reported by (Ratcliffe et al., 2000) and similar to overexpression of P2A:B56α (Li et al., 2001)].