

## Original Article

# Cell-autonomous signal transduction in the *Xenopus* egg Wnt/ $\beta$ -catenin pathway

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Wnt proteins are thought to bind to their receptors on the cell surfaces of neighboring cells. Wnt8 likely substitutes for the dorsal determinants in *Xenopus* embryos to dorsalize early embryos via the Wnt/ $\beta$ -catenin pathway. Here, we show that Wnt8 can dorsalize *Xenopus* embryos working cell autonomously. Wnt8 mRNA was injected into a cleavage-stage blastomere, and the subcellular distribution of Wnt8 protein was analyzed. Wnt8 protein was predominantly found in the endoplasmic reticulum (ER) and resided at the periphery of the cells; however, this protein was restricted to the mRNA-injected cellular region as shown by lineage tracing. A mutant Wnt8 that contained an ER retention signal (Wnt8-KDEL) could dorsalize *Xenopus* embryos. Finally, Wnt8-induced dorsalization occurred only in cells injected with Wnt8 mRNA. These experiments suggest that the Wnt8 protein acts within the cell, likely in the ER or on the cell surface in an autocrine manner for dorsalization.

**Key words:** endoplasmic reticulum, immunohistochemistry, KDEL, Wnt, *Xenopus*.

## Introduction

Early development of *Xenopus* embryos is established through the interaction of cytoplasmic determinants. Cytoplasmic transplantation studies revealed the presence of a “dorsal determinant” in future dorsal cells of the 16-cell stage embryo (Yuge *et al.* 1990) as well as in the vegetal pole of the fertilized egg (Fujisue *et al.* 1993; Holowacz & Elinson 1993). The dorsal determinant in the vegetal pole is necessary for *Xenopus* dorsal axial development (Kikkawa *et al.* 1996; Sakai 1996). However, the dorsal determinant (DD) alone is

not sufficient because vegetal cortex transplantation resulted in dorsal structure formation only when it is transplanted into the subequatorial region of the embryo (Kageura 1997). These data suggest that the Spemann organizer forms as a result of mixing the dorsal and subequatorial determinants (Sakai 2008). Dorsal endoderm is thought to induce the Spemann organizer during the cleavage stage; however, this induction seems to occur after the end of the cleavage stages (Gurdon *et al.* 1985; Wylie *et al.* 1996; Nagano *et al.* 2000). The “mesoderm inducer” is proposed to be the organizer factor chordin (Vonica & Gumbiner 2007) which is expressed after the cleavage stages. Therefore, we suggest that formation of the organizer is the result of cell autonomous functions of the determinants but not the result of so-called mesoderm induction (Sakai 2008).

Wnt/ $\beta$ -catenin signaling is likely involved in this cell-autonomous process (Moon 2005). Wnt8 is not present in cleavage stage embryos, and its function in normal development is the posteriorization, not the dorsalization, of the embryo (Christian & Moon 1993; Fujii *et al.* 2008). Interestingly, however, Wnt8 mRNA induces a complete secondary body when injected into a ventral blastomere of early *Xenopus* embryos (Christian *et al.* 1991; Smith & Harland 1991). Additionally,

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this molecule acts upstream of  $\beta$ -catenin (Heasman *et al.* 1994), supporting the idea that Wnt8 substitutes for the dorsal determinant (DD). Heasman and colleagues postulated that Wnt5a and Wnt11 work synergistically as the DD (Cha *et al.* 2008). On the other hand, zebrafish Wnt8a has been recently proposed to be the intrinsic dorsal determinant in this species (Lu *et al.* 2011).

Permanent blastula-type embryos (PBEs) are obtained when >60% of the vegetal region (including both the surface and cytoplasm) is ablated from fertilized *Xenopus* embryos. Because PBEs neither have dorsal nor subequatorial determinants, the molecular nature of the latter is VegT mRNA (Zhang *et al.* 1998), they do not form dorsal structures or express dorsal marker genes (Fujii *et al.* 2002, 2008). When Wnt8 and VegT mRNAs were co-injected into a PBE blastomere, it forms dorsal structures and expresses the organizer marker gene *chordin*. However, when the two mRNAs were injected separately, the resulting embryo showed no sign of dorsalization (Katsumoto *et al.* 2004). This finding led us to hypothesize that Wnt8 acts with VegT in a cell-autonomous manner.

Although the dorsal determinant has been proposed to act in a cell-autonomous manner to form the Spemann organizer (Sakai 1996; Nagano *et al.* 2000; Katsumoto *et al.* 2004; Vonica & Gumbiner 2007), Wnt proteins are believed to act in a non-cell autonomous manner on the cell surface (Clevers 2006). It has been shown that Wnt8 proteins are localized at the plasma membrane (Yang-Snyder *et al.* 1996; Takada *et al.* 2006), which supports the idea that secreted Wnt proteins act on the cell surface; however, if Wnt8 is capable of substituting for the dorsal determinant, it is reasonable to hypothesize that the Wnt8 protein acts in a cell-autonomous manner within the cell. Recent studies revealed that FGF8 and Protogenin, thought to act on the cell surface, can be internalized to the nucleus and can act in a cell-autonomous manner (Suzuki *et al.* 2012; Watanabe & Nakamura 2012). To examine the potential cell-autonomous role of Wnt8 in the dorsalization process of *Xenopus* embryos, we focused on subcellular distribution of Wnt8 using immunofluorescence and confocal microscopy.

We hypothesized that we could precisely determine the axis-forming activity of injected Wnt8 mRNA using PBEs as a test system because they do not form the dorsal structure (proboscis) or express dorsal marker genes if they receive neither Wnt8 nor VegT mRNAs. However, they can be dorsalized when these mRNAs are co-injected (Katsumoto *et al.* 2004). Furthermore, because PBEs do not have large vegetal yolk granules, they should facilitate visibility in our immunohistochemical observations.

## Materials and methods

### Experimental animals

We have maintained all animals in accordance with the ARRIVE guidelines on the care and use of experimental animals of Kagoshima University.

### Permanent blastula-type embryos (PBEs)

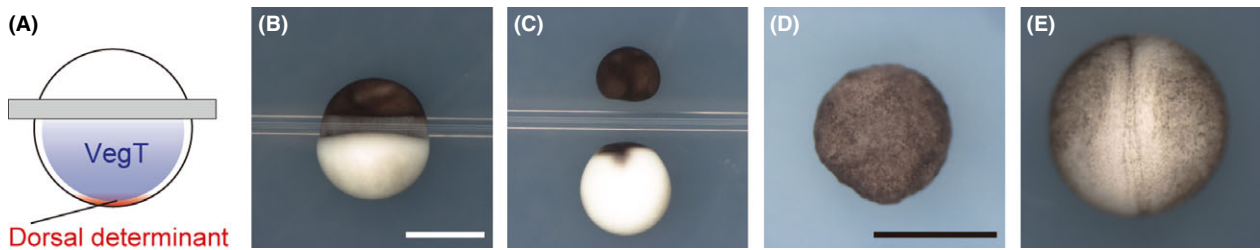
Fertilization and embryo culture were performed as described (Katsumoto *et al.* 2004). PBEs were obtained by ablating >60% of the vegetal region, including the cytoplasm, yolk, and cell membrane (Fujii *et al.* 2002). The denuded egg was inclined 90° off its vertical axis. A glass rod (diameter 300  $\mu$ m, length 2 cm) was placed on the egg to divide it into animal and vegetal fragments (Fig. 1). The PBE derives from the animal part of the embryo, and therefore lacks most of the large yolk platelets. This characteristic facilitated the observation of HA staining.

### Plasmid construction and mRNA synthesis

*Xwnt8-HA* was generated using polymerase chain reaction (PCR). The cDNA was inserted with the HA epitope tag sequence (5'-tatccatcacgatgtaccagattacgca-3'; YPYDVPDYA) at the same position that harbored the Myc epitope tag described by Christian & Moon (1993). The cDNA was subcloned into pCS2+. *Xwnt8-HA-KDEL* was generated using PCR. The cDNA was added at the C-terminus with spacer sequences (5'-agatcgtacaag-3'; RSYK) and KDEL sequences (5'-aaggacgagctg-3') and was subcloned into pCS2+.  $\Delta$ SP-*Xwnt8-HA*, which lacks the predicted signal peptides (2nd-QNTTLFILATL LIFCPFFTASA-23th a.a.), was generated using an inverse PCR technique. Synthetic capped mRNAs were produced using mMessage mMachine SP6 kit (Ambion, AM1340).

### Microinjection

For microinjection, mRNAs were diluted in sterile nuclease-free water containing fluorescent lineage tracers as described below. In the experiments shown in Figures 2 and 5, mRNAs were injected with Alexa Fluor 647 dextran into a single blastomere of 4 to 8-cell stage PBEs with a glass capillary with a diameter of 5–7  $\mu$ m, using a Narishige micromanipulator. For the experiment shown in Figure 7, *Wnt8* mRNA and Oregon Green dextran were injected into one C4 blastomere of normal embryos with regular cleavage



**Fig. 1.** Permanent blastula-type embryo (PBE) as a test system for Wnt8 distribution. (A) Schematic drawing of the cytoplasmic contents of the PBE (upper area). PBEs do not contain both the dorsal determinant and the VegT (endomesodermal determinant). (B–D) Deletion of the vegetal cytoplasm (>60% of the egg surface) with a glass rod results in the formation of the PBE. (B) Just after placing the glass rod. (C) Just after the separation into an animal egg fragment (PBE) and a vegetal egg fragment. (D) A PBE at the neurula stage. The PBE does not form dorsal or endomesodermal structures. (E) A normal embryo at the same stage.

pattern (Kageura 1990). The amounts of injected RNA and lineage tracers are indicated in the respective figure legends.

#### Immunohistochemistry

Permanent blastula-type embryos (PBEs) injected with mRNAs and Alexa Fluor 647 dextran (lineage tracer) were fixed in MEMPFA [4% paraformaldehyde, 100 mmol/L MOPS (pH 7.4), 2 mmol/L EGTA, 1 mmol/L MgSO<sub>4</sub>] for 1 h at 20°C at the 4000-cell stage, just before the gene expression commences (Newport & Kirschner 1982) to avoid possible effects of the newly synthesized transcripts. For the correct timing of the fixation, embryos were continuously scored for each cleavage. Typically (Figs 2, 6), the embryos were dehydrated and stored in methanol at –20°C for at least 3 days. In some cases, the methanol storage step was skipped. Embryos were embedded in OTC compound (Tissue-Tek) and cut into 8- $\mu$ m sections using a cryostat. After treatment with 1%

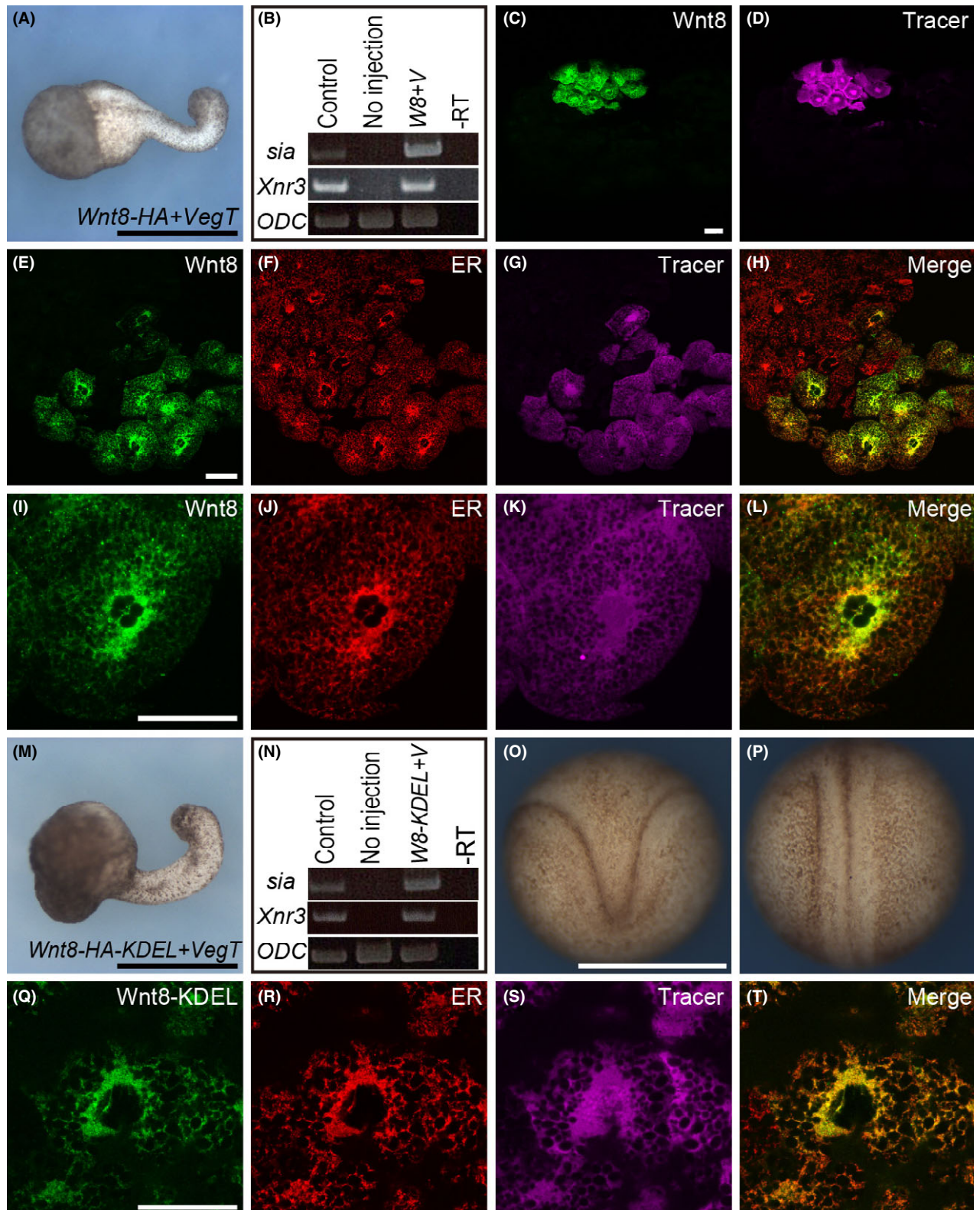
skim milk in phosphate-buffered saline (PBS) for 1 h, the sections were incubated overnight at 4°C in a mixture of the rat anti-HA monoclonal antibody (mAb; 3F10, Roche, 1:200), mouse anti-PDI mAb (ab12225, Abcam, 1:4000), and goat anti-Frzb (sFRP3) Ab (AF192, R&D, 1:2000). Anti-rat IgG-Alexa488 (Invitrogen, 1:250), anti-mouse IgG-Alexa594 (Invitrogen, 1:250), and anti-goat IgG-Alexa594 (Invitrogen, 1:250) were used as secondary antibodies, and samples were incubated for 2 h at room temperature with these antibodies. Observations were performed using a Leica TCS SP2 confocal laser microscope.

#### Preparation of cell lysates and concentration of culture media

We seeded HEK293 cells at a density of  $3.8 \times 10^5$  cells in 60-mm dishes containing modified Eagle's media supplemented with 10% horse serum. Cells were cultured for 24 h. Aspirated media were replaced with 4 mL Opti-MEM (Invitrogen). HEK293

**Fig. 2.** Wnt8 protein is localized in the endoplasmic reticulum (ER) of mRNA-injected cells. (A) A stage-17 permanent blastula-type embryo (PBE) injected with *VegT* (15 pg) and *Wnt8-HA* (3 pg) into a blastomere at the 4-cell stage. Co-injection of *VegT* mRNA is necessary for proboscis formation (Katsumoto *et al.* 2004), which is a marker of dorsalization. Formation of the proboscis (34 out of 34) indicates dorsalization. (B) Reverse transcription–polymerase chain reaction (RT–PCR) analyses for dorsalization markers *siamois* and *Xnr3* expression in control PBEs and PBEs injected with the two mRNAs. ODC serves as loading control. Control, normal embryos; No injection, PBEs with no mRNA injection; *W8+V*, PBEs injected with *Wnt8-HA* (3 pg) and *VegT* (15 pg) mRNAs; –RT, PCR with cDNA synthesized without reverse transcriptase. (C,D) A transverse section of a PBE injected with 250 pg *Wnt8-HA* and 15 pg *VegT* mRNAs containing 0.1% Alexa Fluor 647 dextran into an 8-cell stage blastomere. The sample was fixed and processed for immunostaining 2 h before stage 10. (E–L) Low-magnification (E–H) and high magnification (I–L) images showing the same region of a section. (E,I) Subcellular distribution of Wnt8-HA protein (green). (F,J) PDI immunostaining (red) showing subcellular distribution of ER. (G,K) Alexa Fluor 647 staining (magenta) showing the lineage of the mRNA-injected cells. (H,L) Merged images of Wnt (E, I) and PDI staining (F, J). Yellow regions indicate the overlap of these two molecules. Note the strong fluorescence of Alexa Fluor 647 in the nucleus (G,K), which is connected to the cytosol via the nucleopore, whereas HA and PDI staining was absent in the nucleus (I,J and L). (M) A PBE injected with *VegT* (15 pg) and *Wnt8-HA-KDEL* (10 pg). Proboscises (12 out of 12) formed as shown in (A). (N) RT–PCR for *siamois* and *Xnr3*. (O) An embryo with duplicated axis: This embryo was injected with 15 pg Wnt8-HA-KDEL mRNA into a ventral vegetal blastomere of 8-cell stage embryo. (P) A control embryo at the same stage (stage 17). (Q–T) Wnt8-HA-KDEL protein also overlapped with ER staining. Staining was the same as for the above-mentioned panels for Wnt8-HA (I–L). Bar in A and M, 1 mm. Bar in C for C and D, 100  $\mu$ m. Bar in E–H, 100  $\mu$ m. Bar in I for I–L, 50  $\mu$ m. Bar in O for O–R, 50  $\mu$ m. Bar in O for O and P, 1 mm.





cells were transfected with expression plasmids using FuGENE6 (Roche). Culture media were collected after 24 h (1 day) or 72 h (3 days), and cell lysates were

prepared. Protein concentrations were determined as described previously (Lintern *et al.* 2009). Culture media were clarified to remove debris. Supernatants

were concentrated 35- to 50-fold and stored in aliquots at  $-80^{\circ}\text{C}$ .

#### Western blotting

Total cell lysate protein (5  $\mu\text{g}$ ) and concentrated culture media (an equivalent volume of 500  $\mu\text{L}$  of non-concentrated culture media) were resolved by electrophoresis on 15% sodium dodecyl sulfate–polyacrylamide gels (SDS–PAGE) and probed with the primary anti-HA antibody (Roche, 3F10, 1:1000) and the secondary anti-rat IgG-(Fab)'2(HRP) antibody (Abcam, ab6517, 1:40 000). Proteins were detected using a 1:10 dilution of the reagent mixture provided with the ECL Prime System (GE Healthcare).

#### In situ hybridization combined with histochemistry for lineage tracing

*Wnt8* mRNA (10–20 pg/nL) and Oregon Green dextran (1%) in 1 nL of distilled water (DW) were injected into a C4 blastomere of the 32-cell embryo (Nakamura & Kishiyama 1971). The embryos were fixed 30–60 min before stage 10 in MEMFA overnight at room temperature, washed thoroughly, and stored in 100% methanol ( $-20^{\circ}\text{C}$ ) for at least 3 days. Whole-mount *in situ* hybridization was carried out following the method of Sive *et al.* (Sive *et al.* 2000), without proteinase K treatment. After *in situ* hybridization, embryos were cut vertically to facilitate the immunostaining. The cell lineage tracer Oregon Green dextran was detected by immunostaining using anti-fluorescein-AP Fab fragments (Roche) and Fast Red (Roche) following the method of Koga *et al.* (2007).

#### RT–PCR

Total RNA was extracted from a single embryo or three PBEs using the SV Total RNA Isolation System (Promega). cDNA was synthesized from total RNA (3.1  $\mu\text{g}$ ) using Ready-To-Go You-Prime First-Strand Beads (GE Healthcare). Each PCR reaction was performed using 1  $\mu\text{L}$  of the reverse transcription reaction product as template, 0.25 U rTaq DNA polymerase (Takara Bio, Shiga, Japan), 10  $\mu\text{mol/L}$  each primer, and 2.5 mmol/L dNTPs in volume of 10  $\mu\text{L}$  of 1 $\times$  PCR buffer. The denaturation step proceeded at  $95^{\circ}\text{C}$  for 30 s. The annealing temperatures were  $65^{\circ}\text{C}$  for *ODC*,  $58^{\circ}\text{C}$  for *siamois*, and  $54^{\circ}\text{C}$  for *Xnr3*. Annealing proceeded for 30 s before final extension at  $72^{\circ}\text{C}$  for 30 s. There were 23 cycles for *ODC*, 25 cycles for *siamois*, and 30 cycles for *Xnr3*. PCR products were analyzed on a 6% acrylamide gel. The primers were as

follows: *ODC*, 5'-GCAAAGCCATTGTGAAGACTCTCTCATTTC-3' and 5'-AAGCTTTGCATTTCGGGTGATTCC TTGCCAC-3'; *siamois*, 5'-AGACATGACCTATGAGGCTG-3' and 5'-AGTCAGTTTGGGTAGGGTAGG-3'; and *Xnr3*, 5'-TAATCTGTTGTGCCGATCCA-3' and 5'-ATCAATGTTGCCCTTTTTC-3'.

## Results

#### *The Wnt8-HA protein is localized in the endoplasmic reticulum (ER) of mRNA-injected cells*

We injected HA-tagged *Wnt8* mRNA (*Wnt8-HA*) together with *VegT* mRNA into a PBE blastomere (Fig. 1) (Sakai 1996; Fujii *et al.* 2002). PBEs do not form dorsal structures (Fig. 1D) or express dorsal genes, but these embryos do form dorsal structures and express dorsal genes following injection of *Wnt8* and *VegT* (Katsumoto *et al.* 2004). Because HA-tagged *Wnt8* dorsalized PBE (Fig. 2A,B), HA appears to have no inhibitory effects on *Wnt* activity.

Immunohistochemistry with anti HA tag showed that HA positive cells are restricted within the *Wnt8* mRNA-injected cells that are assessed by co-injected Alexa Fluor 647 fluorescence (compare 2C and D, 2E and G). It was also shown that *Wnt8* protein was mainly co-localized with the ER (endoplasmic reticulum) marker PDI (compare Fig. 2E and F, I and J). Merged images clearly show that *Wnt8* proteins are mainly localized in the ER (Fig. 2H,L). These results support our hypothesis that *Wnt8* protein could act within the cell, presumably in the ER.

#### *ER-retention signal does not inhibit dorsalization by Wnt8*

To further confirm our hypothesis, we constructed a *Wnt8* construct tagged with the ER-retention signal KDEL (Munro & Pelham 1987; Pelham 1988) (*Wnt8-HA-KDEL*). As we expected, PBEs injected with *Wnt8-HA-KDEL* and *VegT* mRNA expressed *siamois* and *Xnr3* (Fig. 2N), and formed a proboscis (Fig. 2M). Further, *Wnt8-HA-KDEL* generated duplicated axis in normal embryos (Fig. 2O) when injected into a ventral vegetal blastomere of 8- to 16-cell stage embryos (23 out of 23 embryos formed Y-shaped secondary axis). These results indicate that *Wnt8-HA-KDEL* has dorsalizing activity. The dose needed for an identical result was approximately three times higher than *Wnt8-HA*, which might be due to the general inhibitory effect of the additional tag sequence KDEL or *Wnt8-HA-KDEL* might not have dorsalizing activity acting on the cell surface which might be present in *Wnt8-HA*. Upon



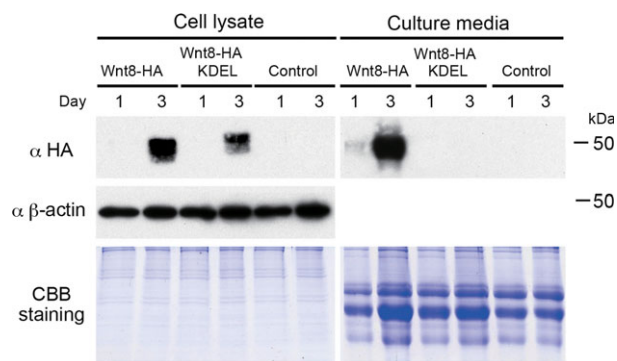
immunofluorescence examination of the injected embryos, Wnt8-HA-KDEL protein was also localized in the ER (Fig. 2Q–T).

#### Wnt8-HA-KDEL is retained within the cell in cell culture

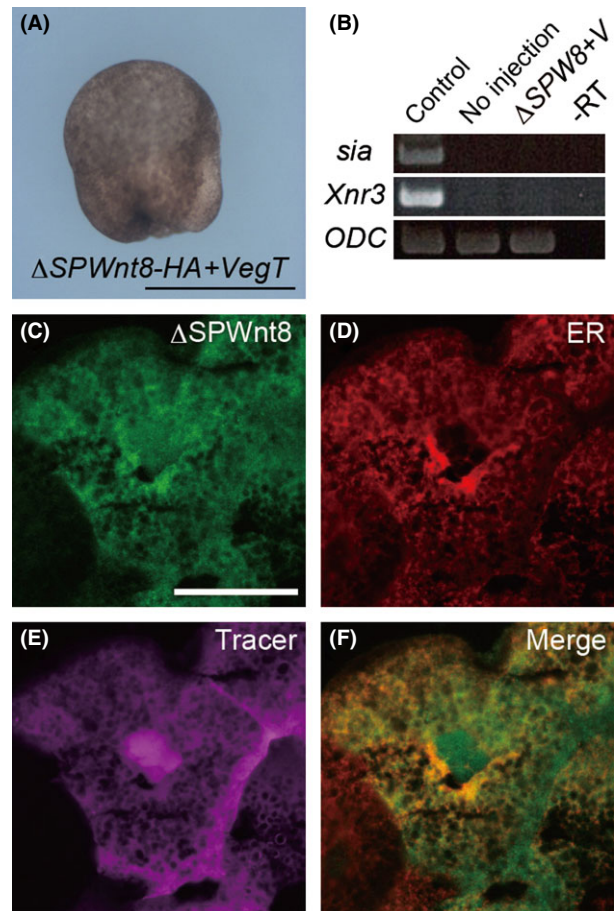
To examine ER retention of Wnt8-HA-KDEL, we analyzed the Wnt8-HA-KDEL protein in culture media from HEK293 cells transfected with Wnt8-HA-KDEL expression plasmids (Fig. 3). Wnt8-HA was observed in both the cell lysate and the culture media 3 days after transfection. In contrast, Wnt8-HA-KDEL was observed in the cell lysate, but not in the culture media 3 days after transfection. These data suggest that the Wnt8-HA-KDEL protein is retained in the ER.

#### The signal peptide is required for dorsalizing activity

Moreover, to test whether entry of the Wnt8 protein into the ER is required for dorsalizing activity, we constructed a mutant Wnt8 that lacks a signal peptide for secretion ( $\Delta$ SP-Wnt8-HA).  $\Delta$ SP-Wnt8-HA was present in the cytoplasm and in the nucleus (Fig. 4C–F) of mRNA-injected cells. Some part of  $\Delta$ SP-Wnt8-HA seems to overlap with ER marker PDI (Fig. 4F). But the merged image show that green area, staining for  $\Delta$ SP-Wnt8-HA, is present, which means that some part of  $\Delta$ SP-Wnt8-HA is localized apart from ER (Fig. 4F).  $\Delta$ SP-Wnt8-HA was also localized in the nucleus (Fig. 4F). We speculated that large part of  $\Delta$ SP-Wnt8-HA left ER after translation is completed.  $\Delta$ SP-Wnt8-HA did not dorsalize PBEs (Fig. 4A,B), sug-



**Fig. 3.** Wnt8-HA-KDEL protein is retained in the cell in cell culture. Plasmid constructs of Wnt8-HA or Wnt8-HA-KDEL were transfected into HEK293 cells. Proteins (40  $\mu$ g) from total cell lysates were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and examined by Western blotting using the primary anti-HA antibody.  $\beta$ -actin was used as a loading control (cell lysates). Coomassie brilliant blue (CBB) staining of acrylamide gels was used as a loading control (cell lysates and culture media).



**Fig. 4.** Wnt8 lacking a signal peptide ( $\Delta$ SP-Wnt8-HA) did not cause dorsalization. (A)  $\Delta$ SP-Wnt8-HA-injected PBEs do not form dorsal structures. Proboscises were not observed (0 out of 10). (B) Reverse transcription–polymerase chain reaction (RT–PCR) analyses for dorsalization markers *siamois* and *Xnr3* expression in control PBEs (no injection) and PBEs injected with  $\Delta$ SP-Wnt8-HA and *VegT*. (C–F)  $\Delta$ SP-Wnt8-HA (green) was broadly expressed in the cytosol of the injected cells. Note that strong HA staining (C) was observed in the nucleus, whereas ER staining was not found in the nucleus (D), suggesting that  $\Delta$ SP-Wnt8-HA is in the cytosol and not in the ER.

gesting that ER entry of the Wnt8 protein is required for its activity.

#### Localization of Wnt8 at the cell boundaries is revealed in the absence of methanol treatment, although its distribution is restricted to the area of mRNA injection

Although our experiments revealed that Wnt8 was localized in the ER, it has been accepted that Wnt proteins are localized mainly at the cell boundaries (Yang-Snyder *et al.* 1996; Takada *et al.* 2006). The main difference in the protocols between the previous studies and the present study was methanol treatment

**Fig. 5.** Wnt8 is localized at the cell boundaries in the absence of methanol treatment, but is restricted within the tracer injected domain. Wnt8-HA or Wnt8-HA-KDEL proteins were detected by HA-immunostaining (green), and the lineage of mRNA-injected cells was shown by Alexa Fluor 647 dextran (magenta) as in Figure 2. Samples were not treated with methanol in these experiments. (A–F) A *Wnt8-HA* injected sample. (A) Low magnification image of Wnt8-HA staining. Note that weak but substantial staining within the cell, likely in the endoplasmic reticulum (ER), was also detected. (B) Alexa Fluor 647 staining (magenta) showing the lineage of the mRNA-injected cells. (C) Composite image of A and B. Alexa Fluor 647 (lineage tracer) is indicated in the red and blue channel (thus shown in magenta) whereas Wnt8-HA is represented in the green channel. (D–F) High magnification view of A–C. (G–L) *Wnt8-HA-KDEL* injected sample. Staining within the cell is stronger as compared to Wnt8-HA (A–F). (G) A *Wnt8-HA-KDEL* injected sample. (H) Alexa Fluor 647 staining (magenta) (I) Composite image of G and H. (J–L) High magnification view of G–I. White arrowheads indicate the border of the lineage-labeled/negative cells. Bar in A for A–C and G–I, and bar in D for D–F and J–L: 100  $\mu\text{m}$ .

after fixation. In our protocol, embryos were stored in methanol for longer than 3 days for permeabilization (see Materials and methods), whereas methanol was not used or only briefly used in the previous experiments. Wnt proteins in the ER may not have been detected effectively due to insufficient permeabilization. According to our protocol, most parts of Wnt8 protein on the cell surface may have been lost by the long-term storage in methanol. Therefore, we analyzed the distribution of Wnt8 without using methanol (Fig. 5). Wnt8 protein was detected mainly at the cell boundaries in the absence of methanol treatment (Fig. 5A–F). As shown in previous studies (Yang-Snyder *et al.* 1996; Takada *et al.* 2006), weak but substantial staining within the cell, likely in the ER, was also detected (Fig. 5A,D). Most importantly, Wnt8 staining was restricted to the region of mRNA injection as shown by the lineage tracer (compare Fig. 5A,B). High-resolution images also show the absence of HA staining in tracer-negative cells (compare Fig. 5D,E).

Furthermore, we examined the distribution of the Wnt8-KDEL protein in the absence of methanol treatment (Fig. 5G–L). As compared to Wnt8, the Wnt8-KDEL protein tends to be localized inside of the cell (compare Fig. 5A and G, D and J) although the staining was stronger at the cell boundary (Fig. 5G,J). Wnt8-HA-KDEL staining also did not exceed outside of the lineage positive cells (Fig. 5G,H,J,K).

#### *Co-injection of Frzb mRNA and Wnt8 or Wnt8-KDEL mRNA displayed distinct distributions*

We co-injected Frzb mRNA, which is an inhibitor of Wnt8 (Leyns *et al.* 1997; Wang *et al.* 1997) and examined whether Frzb changes the distribution of Wnt8-HA, using our methanol method (Fig. 6). Interestingly, Frzb (Fig. 6B) and Wnt8-HA (Fig. 6C) were both observed predominantly at the cell boundaries and were even observed in the lineage-negative cell domain (Fig. 6D,E). Since Wnt8-HA is localized in the ER when the samples were treated with methanol (Fig. 2E,H,I,L), the distribution at the cell boundary should be the

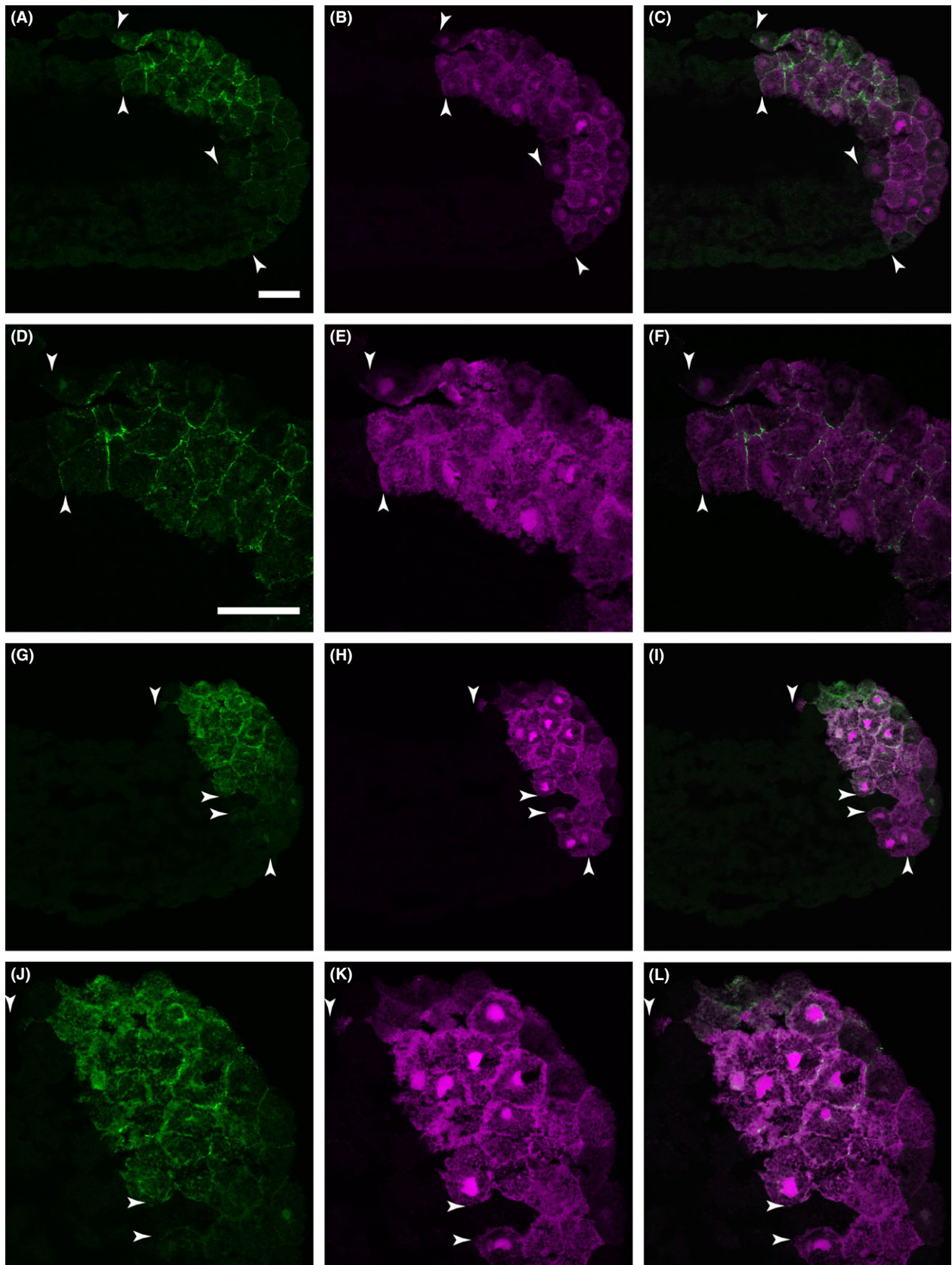
effect of Frzb, which moves out of the cell (Mii & Taira 2009). Frzb was found on the cell surface of the whole PBE while Wnt8 was noted two to three cells apart from the lineage-labeled cells (compare Fig. 6B and C, D and E). These results indicate that these proteins are secreted outside of the mRNA-injected cells, and that using the methanol method of immunohistochemistry we can visualize the secreted proteins.

On the contrary, when *Wnt8-HA-KDEL* was co-injected with *Frzb*, the resulting PBE showed distinct staining pattern (Fig. 6G–L). Although Frzb was found at the cell boundaries throughout the PBE, some staining still existed within the cell of the lineage positive region (Fig. 6H). Wnt8-HA-KDEL was seen only inside of the lineage positive cells (Fig. 6I,K). The Frzb staining at the cell boundaries does not overlapped with Wnt8-HA-KDEL (Fig. 6L, note red Frzb staining outside of the green Wnt8-HA-KDEL staining) suggesting that the Frzb staining resides outside of the cell while Wnt8-HA-KDEL resides inside of the cell and Frzb does not alter the distribution pattern of Wnt8-HA-KDEL. These data suggest that the Wnt8-HA-KDEL protein behaves differently from the Wnt8-HA protein: the former tends to stay within the cell in which the mRNA was injected, presumably in the ER while the latter going out of the cell with Frzb.

#### *Dorsalization occurs in cells following Wnt8 mRNA injection*

Immunohistochemical analyses of the Wnt8 protein revealed that Wnt8 resides in the ER and on the cell surface. Because Wnt8-KDEL can dorsalize *Xenopus* embryos as described above and resides predominantly in the ER, we hypothesized that Wnt8 protein in the ER is functioning as the dorsalizing factor.

Therefore, we examined whether dorsalization occurs in cells adjacent to cells that were injected with *Wnt8* mRNA. To follow the lineage of the injected blastomere, we used Oregon Green dextran (Koga *et al.* 2007). A mixture of *Wnt8* mRNA and Oregon Green dextran was injected into a C4 blastomere of a normal





embryo (Nakamura & Kishiyama 1971) at the 32-cell stage, reared until 30–60 min before the onset of gastrulation (30–60 min before stage 10), and the embryo was fixed overnight in MEMFA. We examined *chordin* expression as a marker of the organizer (Sasai *et al.* 1994) using *in situ* hybridization. *Chordin* is not the first gene to be expressed in the organizer domain; however, expression of this factor is readily observed just before gastrulation (Vonica & Gumbiner 2007). After *in situ* hybridization, samples were processed for Oregon Green immunostaining using Fast Red and then bleached. In general, the expression pattern of *chordin* overlapped with the Fast Red staining (Fig. 7). This overlapping pattern should be reliable because the majority of embryos showed a very precise overlapping pattern. Punctate *chordin* perinuclear staining was observed exclusively in cells injected with *Wnt* mRNA based on Fast Red immunostaining against Oregon Green (Fig. 7A,B). Distribution pattern of the nuclei, which was stained with Hoechst 33248 (Fig. 7C), facilitated observation at the single cell level. The nuclei adjacent to the *chordin*- and lineage tracer-positive cells did not exhibit a *chordin* signal in the absence of Fast Red staining (Fig. 7B–D).

We repeated these experiments using 21 batches of embryos ( $n = 463$ ). Although some embryos ( $n = 160$ ) were not stained clearly, 243 embryos exhibited an overlapping pattern whereas in 60 embryos, *chordin* was expressed outside of the *Wnt*-8-injected cells. If the latter is due to *Wnt* signaling at the cell surface, *chordin*-positive cells should be adjacent to the injected cells because *Wnt* protein distribution was restricted to the *Wnt* mRNA-injected cells and their descendants (Figs 2C–F, 5A,B). *Chordin* expression outside of the lineage usually showed a cluster-like pattern or a broad spreading pattern, suggesting that expression was due to leakage of the injected *Wnt* mRNA through a cytoplasmic bridge or that *chordin*-induced *chordin* expression (Nagano *et al.* 2000) occurred after the mid-blastula transition (Newport & Kirschner 1982).

Even with these data, it is still possible that *Wnt8* acts in an autocrine manner: *Wnt8* may be secreted and act on the “source” cells in an autocrine manner.

## Discussion

The first step of signal transduction generally involves the interaction of a ligand with its receptor on the surface of neighboring cells. Here, we show that the signaling protein *Wnt8* could act in a cell-autonomous manner, in the dorsalizing process of early *Xenopus* embryos. This finding initially appeared curious. How-

ever, if we consider that signaling molecules or ligands are synthesized in the ER and are thereafter transported to the cell surface, and that the receptor is also synthesized in the ER and incorporated into the ER membrane before being translocated to the plasma membrane, it is reasonable that these two molecules interact within the ER. The outer space of the ER is cytoplasm, and the inner space of the plasma membrane is also cytoplasm. The cytoplasmic domain of the *Wnt8* receptor on the cell membrane or on the ER membrane might transduce the signal to the cytoplasm.

We first analyzed the distribution of *Wnt8* protein in *Xenopus* PBEs which had been injected with *Wnt8* and *VegT* mRNA. *Wnt8* was distributed within the domain of the descendants of *Wnt8* mRNA-injected cells. Furthermore, *Wnt8* was mainly distributed in the ER. It has been accepted that *Wnt* proteins are distributed at the cell boundaries, presumably on the cell surface (Yang-Snyder *et al.* 1996; Takada *et al.* 2006). We used methanol to permeabilize the embryo, which was not used in the previous studies. Owing to permeabilization, we could see inside the cell more clearly, and we could show that *Wnt8* is distributed within the cell in the early stage of *Xenopus* embryos. We also confirmed that *Wnt8* was localized at the cell boundaries in the absence of methanol treatment. These findings indicate that *Wnt8* is distributed in the ER and on the cell boundaries.

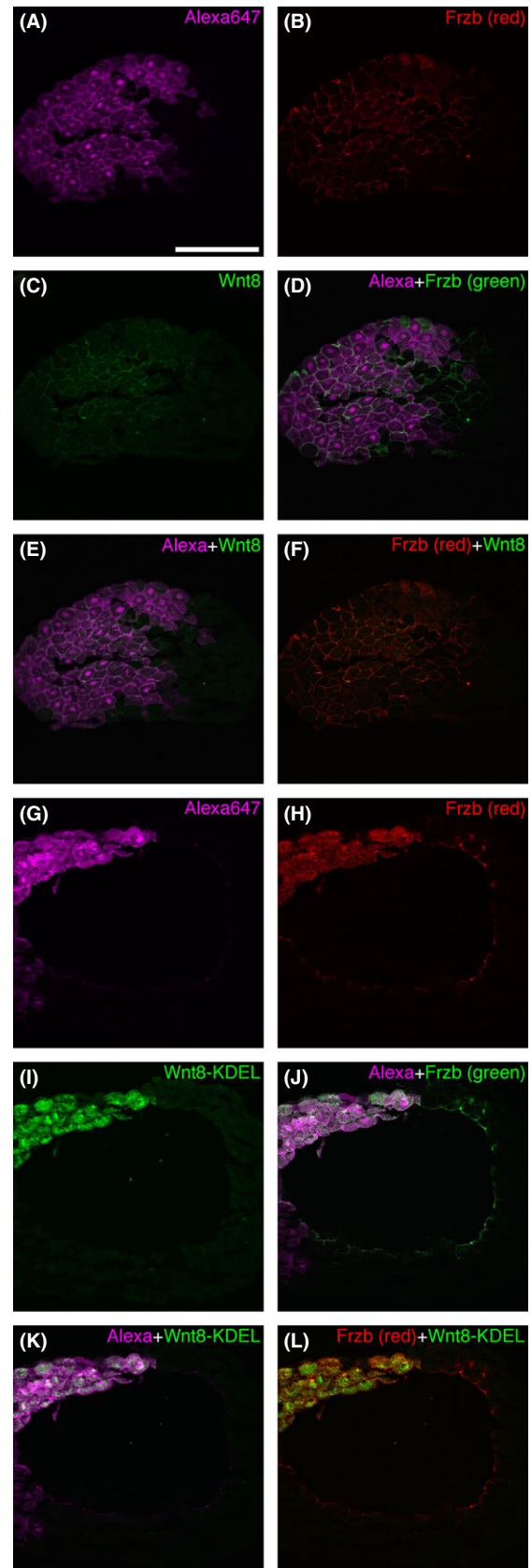
It should be noted that the subcellular localization of the *Wnt* proteins was not analyzed with lineage tracers in previous studies. In the present study, however, we used a lineage tracer to demonstrate that the *Wnt8* protein is distributed within the domain where *Wnt8* mRNA was injected. We used an artificial embryoid PBE to facilitate observation of dorsalization, since we can assay dorsal gene expression using this system easily: PBEs never express dorsal gene while co-injection of *Wnt8/VegT* and *Wnt8-KDEL/VegT* resulted in the expression of *siamois* and *Xnr3*. It should be emphasized that when *Wnt8* and *VegT* are injected separately into the PBE, it neither formed dorsal structure nor expressed an organizer marker *chordin* (Katsumoto *et al.* 2004).

Co-injection of *Frzb* and *Wnt8*-HA or *Wnt8*-HA followed by the immunostaining with the present methanol method revealed interesting results. In both cases, *Frzb* was found on the cell surface throughout the embryo. Further, *Wnt8*-HA and *Wnt8*-HA-KDEL showed distinct pattern of localization. *Wnt8*-HA was localized overlapping with *Frzb* at the cell boundaries and in the lineage tracer-negative cell domain. *Wnt8*-HA may have been transported by *Frzb* to be localized predominantly to the cell surface, and secreted. It has

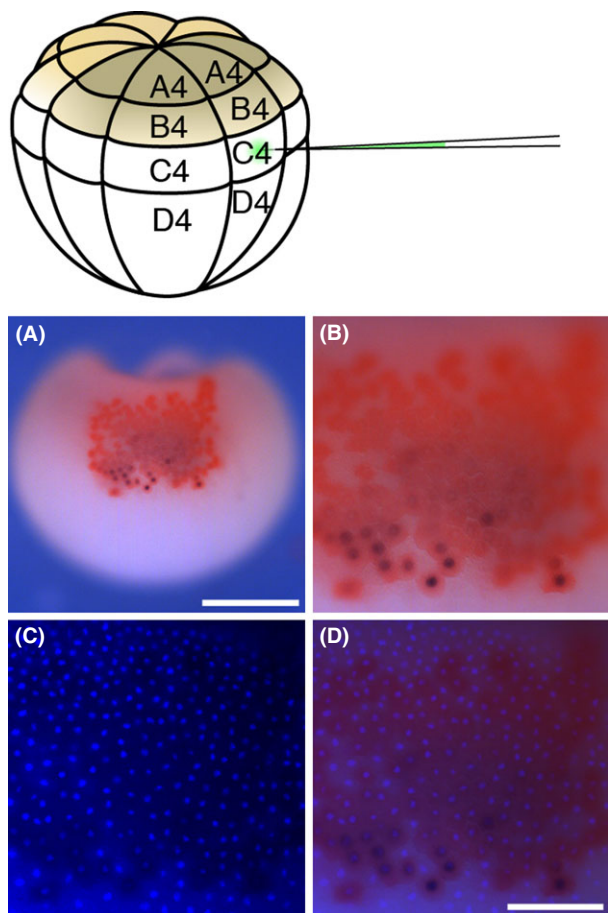
been reported that sFRPs transports and enhance the diffusion of Wnts (Mii & Taira 2009). On the contrary, Wnt8-KDEL was retained within the cell. In the latter case, Frzb was also retained within the mRNA-injected cells.

It has been reported that signal peptide is required for the activity of cWnt3a (Narita *et al.* 2007). Our study revealed that the absence of the signal peptide inhibits dorsalization by Wnt8 protein. Wnt8 protein lacking signal peptide accumulated in the cytoplasm rather than the ER. These results suggest that Wnt8 must enter into ER to act in the dorsalization process.

The presence of Wnt8 in the ER may be more important than Wnt8 on the cell boundaries for dorsalizing activity. First, Wnt8 that is deleted of signal peptide did not have dorsalizing activity. Second, even when ER retention signal KDEL was attached to Wnt8, Wnt8 maintained its activity to dorsalize *Xenopus* embryos. Further, the distribution of Wnt8-KDEL did not extend to cells neighboring the descendants of the



**Fig. 6.** Co-injection of *Frzb* (500 pg) and *Wnt8-HA* (250 pg) or *Wnt8-HA-KDEL* (250 pg) mRNAs. *Frzb* and *Wnt8-HA* stains the cell boundaries, outside of the mRNA-injected domain while *Wnt8-HA-KDEL* is restricted in the lineage positive domain. (A–F) Co-injection of *Frzb* with *Wnt8-HA*. Methanol was used for immunohistochemistry (see Materials and methods). (A) Alexa Fluor 647 staining (magenta) showing the lineage of the mRNA-injected cells. (B) *Frzb* staining is seen predominantly at the cell boundaries, even outside of the mRNA-injected domain. (C) Staining for *Wnt8-HA*. *Wnt8-HA* staining was also at the cell boundaries but it diffused less extensively as compared to *Frzb*. (D) Composite image of A and B. Alexa647 is in the red and blue channel (thus shown in magenta) and originally red *Frzb* staining is represented in the green channel. Note that green *Frzb* staining is observed at the cell boundaries of both the lineage-injected domain and the lineage-negative domain, but not overlapped with the lineage tracer which represents inside of the cell. (E) Composite image of A and C. Note some cell boundaries outside of the tracer-positive region are stained in green. (F) Composite image of B and C. These images were from methanol-treated samples. These results indicate that methanol treatment does not eliminate secreted proteins. *Wnt8* can be detected on the cell surface when it is secreted. (G–L) Co-injection of *Frzb* with *Wnt8-HA-KDEL*. (G) Alexa Fluor 647 staining (magenta). (H) *Frzb* is seen both within the lineage positive cells and cell boundaries throughout the PBE. (I) *Wnt8-HA-KDEL* staining (green) is restricted in the lineage positive cells. (J) Composite image of G and H. (K) Composite image of G and I. Note that the outer region of the lineage positive cells is colored magenta. (L) Composite image of H and I. Note that the outer region of the lineage positive cells is colored red, suggesting that *Frzb* exists on the surface while *Wnt8-HA-KDEL* is absent on the cell surface. Bar in A for A–L, 500  $\mu$ m.



**Fig. 7.** *Chordin* is expressed only in *Wnt8* mRNA-injected cells. (Top) Schematic drawing of the injection of *Wnt8*-HA mRNA and Oregon Green dextran. Normal embryos with a typical cleavage pattern were selected for injection. *Wnt8*-HA mRNA and Oregon Green dextran were injected into a C4 blastomere. The embryo was fixed 30–60 min before the onset of gastrulation and processed for *in situ* hybridization combined with histochemistry for lineage tracing (see Materials and methods). (A) Lateral view of an embryo showing the injected area. Expression of *chordin* is shown as dark blue dots, whereas lineage tracing by immunostaining of Oregon Green dextran is shown in red. (B) Enlarged image of A. (C) Counterstaining with Hoechst 33258. Note that the distribution pattern of nuclei precisely matches that of *chordin* expression. (D) The nuclei pattern can be superimposed on the image and therefore can be used as a single-cell level map. Bar in A, 500  $\mu\text{m}$ . Bar in D for B–D, 200  $\mu\text{m}$ .

injected cells. Therefore, Wnt8-KDEL in the ER might be sufficient for dorsalization. Upon co-injection with Frzb, the KDEL-tagged Wnt8 was not found on the cell surface (Fig. 6I) while Wnt8-HA was found predominantly on the cell surface (Fig. 6C).

Because Wnt8 protein is observed on the cell surface, we could not rule out the possibility that Wnt8 acts on the cell surface of neighboring cells to induce

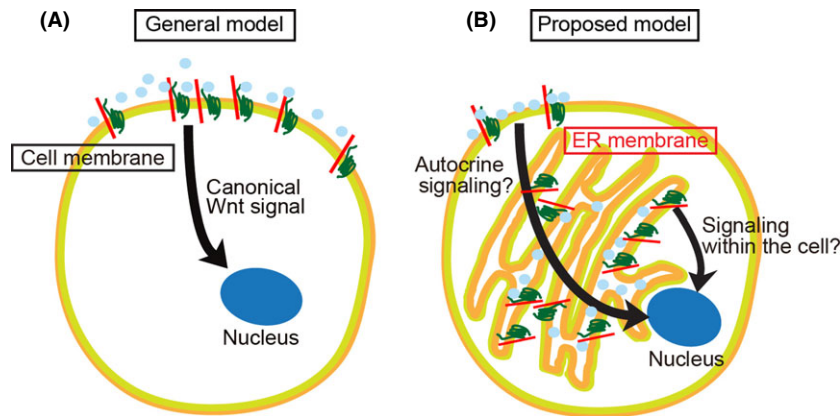
dorsal gene expression. Therefore, we examined *chordin* expression combined with lineage tracing to investigate this possibility. Dorsalization occurred only in cells that were injected with *Wnt8* mRNA. Therefore, the present study, together with previous studies describing the cell-autonomous functions of Wnt/ $\beta$ -catenin pathway molecules (Logan *et al.* 1999; Weitzel *et al.* 2004; Itoh *et al.* 2005; Vonica & Gumbiner 2007), reveals that the initial step of the Wnt/ $\beta$ -catenin pathway in early development occurs in a cell autonomous manner.

In the canonical Wnt pathway, important downstream events are known to occur in the nucleus.  $\beta$ -catenin and Xdsh are intracellular components of the Wnt/ $\beta$ -catenin pathway. The first visible sign of this pathway in *Xenopus* development is enrichment of  $\beta$ -catenin in the nucleus on the dorsal side of the embryo. Xdsh is thought to act upstream of  $\beta$ -catenin (Clevers 2006) and in order to promote dorsalization, Xdsh should reside in the nucleus (Itoh *et al.* 2005). Given that these molecules function in the nucleus, the first signal should therefore initiate from near the nucleus. Because extensive ER membrane exists around the nucleus, signaling through the ER membrane is likely very efficient. In the present study, the Wnt8 protein was found predominantly in the ER near the nucleus, suggesting that the canonical Wnt pathway in this system signals via the ER membrane. Recently, Wnt signaling was reported to require endocytosis (Bellen & Seto 2006; Blitzler & Nusse 2006; Yamamoto *et al.* 2006), and Wnt proteins outside the cell were proposed to be internalized via endocytosis and function in the endosome (Niehrs & Boutros 2010).

Multicellular organisms evolved from unicellular organisms. Signaling in general may have originally evolved in a unicellular organism where “neighboring cells” are not present. Intracellular signaling is noticed in unicellular eukaryote yeast cells (Pahl 1999). Therefore, signaling within the cell, as presented here, may also occur elsewhere, including unicellular organism. Signal transduction pathways other than Wnt/ $\beta$ -catenin pathway may also initiate within the cell. Additional studies in the future should further explore this notion.

In the general canonical Wnt pathway model, Wnt8 protein is secreted from the source cell and the Wnt co-receptors (He *et al.* 1997) that reside on the cell surface of the surrounding cells receive this ligand (Fig. 8A). In the present study, Wnt8 protein was found in the mRNA-injected cells and *chordin*, a sign of dorsalization, was found only in these cells. We conclude that Wnt8 protein, presumably in the ER, has dorsalizing activity in *Xenopus* embryos.





**Fig. 8.** Two models for Wnt signaling. (A) General model. Wnt (light blue) is secreted on the plasma membrane where it interacts with the Wnt receptors (red and green). (B) Our new model. Wnt signaling occurs cell autonomously. It may act within the cell and/or on the cell surface in an autocrine manner.

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## Author's contribution

EM performed the embryological and molecular biological studies and drafted the manuscript. TN performed the molecular biological and immunohistochemical studies and drafted the manuscript. YN carried out the embryological and molecular biological studies. HK participated in the *in situ* hybridization experiments with lineage tracing. AS participated in the immunological studies. SN participated in the design of the study. MS performed the embryological study, conceived the study, and participated in its design and coordination, as well as helped draft the manuscript. All authors read and approved the final manuscript.

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