CORRECTION

Loss of *Porcupine* impairs convergent extension during gastrulation in zebrafish

Qiuhong Chen, Ritsuko Takada and Shinji Takada

There was an error published in *J. Cell Sci.* 125, 2224–2234.

In Fig. 2, panel R was inadvertently duplicated and displayed as panel S. Panel S has been replaced with the correct panel in the figure reprinted below. There are no changes to the figure legend, which is accurate. This error does not affect the conclusions of the study.

![Figure](image-url)

We apologise to the readers for any confusion that this error might have caused.
Loss of Porcupine impairs convergent extension during gastrulation in zebrafish

Qiuhong Chen¹, Ritsuko Takada¹ and Shinji Takada¹,²,*

¹Okazaki Institute for Integrative Bioscience and National Institute for Basic Biology, National Institutes of Natural Sciences, Okazaki, Aichi 444-8787, Japan
²Department of Molecular Biomechanics, The Graduate University for Advanced Studies (SOKENDAI), Okazaki, Aichi 444-8787, Japan

*Author for correspondence (stakada@niib.ac.jp)

Summary

Porcupine (Porcn), an O-acyltransferase located in the endoplasmic reticulum (ER), is required for lipidation of Wnt proteins to enable their trafficking from the ER in mammalian cell culture. However, it is unclear whether Porcn is required for trafficking of all members of the Wnt family. In this study, we investigated the function of Porcn in zebrafish embryos. We identified two zebrafish homologs of porcupine, porcn and porcupine-like (porcn-l). Zebrafish porcn, but not porcn-l, restores secretion of Wnt proteins in porcn-deficient mouse L cells. Morpholino-mediated knockdown of porcn in zebrafish embryos impairs convergence and extension (CE) during gastrulation without changing embryonic patterning. Moreover, porcn interacts genetically with wnt5b and wnt11 in regulating CE. By contrast, porcn-deficient embryos do not exhibit phenotypes caused by failure in canonical Wnt signaling, which is activated by several Wnt ligands, including Wnt3a. Furthermore, expression of genes regulated by the canonical Wnt signaling pathway is not perturbed in knockdown embryos relative to that in controls. Although the trafficking and lipidation of ectopically expressed zebrafish Wnt5b and mouse Wnt5a are impaired in porcn-deficient embryos, those of ectopically expressed Wnt3a are less or not affected. In addition, the secretion of Wnt5a is inhibited by less Porcn inhibitor than that of Wnt3a in HEK293T cells. Thus, a decrease of Porcn activity does not equivalently affect trafficking and lipidation of different Wnt proteins in zebrafish embryos and in cultured mammalian cells.

Key words: Wnt, Secretion, Lipidation, Porcupine, Convergent extension, Zebrafish

Introduction

Wnt proteins constitute a large family of secreted signal proteins that play roles in embryogenesis and tissue homeostasis. They require specific machinery for their secretion; for example, Wntless/Evi/Sprinter, a seven-pass membrane protein, is required for intracellular transport of Wnt proteins (Bänziger et al., 2006; Bartscherer et al., 2006; Goodman et al., 2006). After release of Wnt proteins into the extracellular space, Wntless/Evi/Sprinter is recycled from the plasma membrane back to the Golgi by the retromer complex (Prasad and Clark, 2006; Coudreuse et al., 2006; Franch-Marro et al., 2008; Port et al., 2008; Belfakaya et al., 2008; Yang et al., 2008; Pan et al., 2008). Even before the association with Wntless/Evi/Sprinter, Wnt proteins are modified with a fatty acid, palmitoleate (C16:1), at a serine residue in the endoplasmic reticulum (ER) (Takada et al., 2006; Coombs et al., 2010). The amino acid sequence around this serine residue is highly conserved in the Wnt family, and palmitoleoylation might therefore occur on most Wnt proteins. The serine residue is required for secretion of Wnt proteins from mammalian culture cells and in vertebrate embryos (Takada et al., 2006; Galli et al., 2007). However, in the case of Wingless, the Drosophila ortholog of Wnt1, this serine is not required for secretion from Drosophila S2 cells, but for the function of the protein (Franch-Marro et al., 2008). Thus, the role of the palmitoleoylation might differ for different Wnt proteins or in different cellular contexts.

Palmitoleoylation requires an enzyme, Porcupine (Porcn), a member of the family of membrane-bound O-acyltransferases (MBOATs). The Porcn gene was first identified as a segment polarity gene, similar to wingless, in Drosophila and is evolutionally conserved from worms to mammals (Siegfried et al., 1994; Kadowaki et al., 1997; Thorpe et al., 1997; Rocheleau et al., 1997; Tanaka et al., 2000; Hofmann, 2000). Porcn proteins localize to the ER and are required for Wnt trafficking from the ER in mammalian cells and probably in Drosophila embryos. Porcn overexpression promotes lipid modification of Wnt1 and Wnt3a and is required for CE. By contrast, porcn-deficient embryos do not exhibit phenotypes caused by failure in canonical Wnt signaling, which is activated by several Wnt ligands, including Wnt3a. Moreover, expression of genes regulated by the canonical Wnt signaling pathway is not perturbed in knockdown embryos relative to that in controls. Although the trafficking and lipidation of ectopically expressed zebrafish Wnt5b and mouse Wnt5a are impaired in porcn-deficient embryos, those of ectopically expressed Wnt3a are less or not affected. In addition, the secretion of Wnt5a is inhibited by less Porcn inhibitor than that of Wnt3a in HEK293T cells. Thus, a decrease of Porcn activity does not equivalently affect trafficking and lipidation of different Wnt proteins in zebrafish embryos and in cultured mammalian cells.
actually required for all Wnt signaling in vivo, or whether such a requirement is equivalent for all members of the Wnt family.

To investigate these questions, we used zebrafish as a model system and examined the effects of defects in Porcn function on Wnt signals in early embryonic stages because the roles of Wnt signals have been precisely characterized in early zebrafish embryos. Porcn-deficient embryos specifically exhibited defects in convergent-extension (CE) movement, which requires non-canonical Wnt signaling. Surprisingly, these embryos showed no sign of reduction in canonical Wnt signaling. Consistent with this array of phenotypes, trafficking of zebrafish Wnt5b and mouse Wnt5a, but not zebrafish and mouse Wnt3a, was specifically impaired in Porcn-deficient embryos. Thus, Porcn is required for the proper function and trafficking of at least one Wnt protein during early developmental stages of zebrafish, but a decrease of Porcn does not equivalently affect the trafficking and lipidation of different Wnt proteins in these embryos.

**Cellular function and developmental expression of zebrafish Porcn**

We screened a zebrafish genome database, found two genes encoding proteins structurally similar to Porcn in other species and tentatively designated them porcn1 and porcn2 (Fig. 1A). To assess whether these two zebrafish porcn homologs actually encode proteins with Porcn function, we tested their abilities to restore Wnt3a secretion in a mouse L cell clone in which exogenous mouse Wnt3a had been expressed and endogenous Porcn activity had been stably reduced by siRNA (Takada et al., 2006). Because we found that Wnt5a also required porcn for its secretion from L cells (supplementary material Fig. S1),

![Diagram of phylogenetic analysis of porcn-related genes in zebrafish with Porcn genes in other species.](A) Phylogenetic analysis of two porcn-related genes in zebrafish with Porcn genes in other species. The tree was constructed on the basis of percentage identity at the amino acid level. The GenBank accession numbers for the sequences listed above are the following: Drosophila (#AAB18892), Caenorhabditis elegans (#AAC47728), Homo sapiens (NP_982301), Mus musculus (#NP_076127), Xenopus tropicalis (#NP_989365) and Bos taurus (#NP_001094678). The zebrafish Porcupine protein sequences are based on the sequence of our clones. (B,C) Myc-tagged Porcn1 (Porcn) but not Porcn2 (Porcn-l) restored the secretion of both Wnt3a and Wnt5a from Porcn-deficient mouse L cells. Porcn-deficient L cells expressing either Wnt3a (B) or Wnt5a (C) were transiently transfected with either control DNA, Myc-tagged zebrafish porcn1 or Myc-tagged zebrafish porcn2, as indicated. The amount of Wnt3a or Wnt5a in the culture medium (secreted Wnts) and that in the cell lysate were examined by western blotting. Expression of Porcn1 and Porcn2 proteins was also monitored. (D–N) Expression of porcn1 (porcn) (D–I) or porcn2 (porcn-l) (J–N) was assessed at the eight-cell stage (D,J), at the shield stage (E,K), at the tail bud stage (F,L), at the ten-somite stage (M), at the 14-somite stage (G), and at 24 h.p.f. (H,I,N). The transverse section through the trunk of embryos stained with porcn1 (porcn) probe indicates expression in the ventral spinal cord (I). All photographs, except that in I, were taken from a lateral view. In E,F,K,L, anterior is top and dorsal is right. n, notochord.
restoration of Wnt5a secretion was also examined. The transient expression of Myc-tagged Porcn1, but not that of Porcn2, efficiently restored the secretion of both Wnt proteins (Fig. 1B,C), indicating that Porcn1, but not Porcn2, had a function comparable to that of mouse Porcn in Wnt secretion. Thus, we considered that porcn1 is the functional homolog of mouse Porcn, and so we refer to porcn1 and porcn2 as porcn and porcupine-like (porcn-l), respectively, hereafter.

Whole-mount in situ hybridization analysis revealed that both porcn and porcn-l transcripts were expressed during early embryogenesis. Both mRNAs were maternally deposited (Fig. 1D,J). Weak and ubiquitous expression of porcn could be observed from the eight-cell stage until the tail-bud stage (Fig. 1D–F). During mid-somitogenesis, the ventral spinal cord and the ventral hindbrain as well as the ventral diencephalons were enriched with porcn RNA (Fig. 1G). By 24 hours post fertilization (h.p.f.), expression of porcn was clearly observed in the dorsal neural tube, where several wnt genes, including wnt1 and wnt3a are expressed. Expression of porcn-l was ubiquitous, stronger in brain regions until 24 h.p.f. (Fig. 1J–N). However, because four splicing variants of Porcn have been identified in mouse and chick, we also examined which variants were expressed in zebrafish embryos, and detected two of them corresponding to mouse PorcnC and PorcnD (supplementary material Fig. S2).

Reduction in Porcn expression results in morphological defects in axis elongation

Next, we examined the role of zebrafish porcn during embryogenesis by using antisense morpholino-mediated knockdown. An antisense morpholino oligo against porcn (porcn MO) was designed to target the sequence around the first methionine codon in porcn. As expected, porcn MO, but not a control MO, which was generated by changing five bases in the porcn MO (5 mis control MO), successfully blocked the expression of porcn-GFP (supplementary material Fig. S3A,B).

Injection of 1.5 ng porcn MO into embryos caused a defect in axis elongation, a phenotype typically observed in embryos that have abnormal CE movement, resulting from a disturbance in non-canonical Wnt signaling (Jessen et al., 2002; Carreira-Barbosa et al., 2003; Topczewski et al., 2001; Kilian et al., 2003; Westfall et al., 2003). The morphants specifically exhibited shortened body axes, laterally expanded notochords and compressed somites (Fig. 2A,B). These defects were apparent in 24 h.p.f. morphant embryos, which displayed a short nodulating notochord and neural tube (Fig. 2C,D). Embryos injected with a higher dosage of porcn MO exhibited severer phenotypes, including a greatly shortened trunk and tail, as well as split body axis or unfinished epiboly (Fig. 2E). A significant number of dead cells was observed in the head and trunk regions of the morphants. However, such cell death was not observed in embryos co-injected with the porcn MO and a p53 MO (Fig. 2F), which is known to reduce apoptosis caused by non-specific

Fig. 2. Abnormalities of zebrafish embryos injected with porcn MO or porcn mRNA. (A,B) Dorsal views of seven-somite-stage embryos and (C–E) lateral views of 24 h.p.f. embryos injected with 5mis control MO (C) or porcn MO (D). A porcn MO-injected embryo with severe defects (E) is also indicated. (F) Embryos injected with porcn MO and p53 MO exhibited short body axis, but reduced cell death in the brain. (G) A lateral view of a porcn mRNA-injected embryo at 24 h.p.f. stage. (H–W) Molecular marker analysis indicates that loss or gain of porcn function disrupts CE without changing dorsal–ventral patterning. Embryos injected with either 1.5 ng of 5mis control MO (H,J,M,O,R,U), 1.5 ng of porcn MO (I,K,N,P,S,V) or 100 pg of porcn mRNA (L,Q,T,W) were stained with ntl and hgg1 (H,I), flh and papc (M,N), myoD (J,L,O,Q), chd (R–T) or bmp2b (U–W) probes. Dorsal views of embryos are shown at the tailbud stage (H–N) and eight-somite stage (O–Q), animal views of embryos are shown at the shield stage with dorsal to the right (R–W). Staining patterns in embryos injected with 5mis control MO were identical to those in normal embryos (data not shown).
effects of MO treatment (Robu et al., 2007); therefore, these cell deaths might not be specifically caused by knockdown of porcn.

Similar, but less severe, phenotypes were observed by injecting two other non-overlapping morpholinos targeting the 5’UTR of porcn (porcn MO’ and porcn MO”) (supplementary material Fig. S4A–D). Moreover, co-injection of two of the three porcn MOs (either porcn MO with MO’ or MO’ with MO”) at low dosage resulted in a synergistic increase in the percentage, 91.4% and 81.44% respectively, of embryos with body elongation defects, whereas either MO alone caused the phenotype in less than 5% of embryos at this dosage (supplementary material Fig. S4E–H). Finally, the defect in axis elongation was rescued, although not completely, by injection of mouse Porcn mRNA, which did not contain the sequence targeted by zebrafish porcn MO (supplementary material Fig. S3C–G). These results indicate that defective elongation of the body axis caused by all these porcn MOs was due to specific interference with the function of Porcn.

We also designed two non-overlapping MOs, porcn-l MO and porcn-l MO’, which targeted the porcn-l translation start and the 5’UTR sequence, respectively, to investigate the role of porcn-l during zebrafish embryogenesis. Although porcn-l MO blocked the expression of porcn-l–GFP (supplementary material Fig. S5A,B), the injection of either MO alone or the combination of both MOs did not cause an obvious abnormal phenotype, even when high doses of MOs were injected (supplementary material Fig. S5C; data not shown). We also confirmed that porcn-l MO did not significantly influence the effect of porcn MOs when these MOs were injected at various doses (supplementary material Fig. S5D-G). Furthermore, our evidence to show that porcn MO did not affect the expression of porcn-l GFP (supplementary material Fig. S3A) excluded the possibility that the defects caused by porcn MO were partly due to a cross targeting of the related gene, porcn-l. Therefore, we considered that porcn-l did not play a significant role in early zebrafish development and that there was no obvious redundancy in function between porcn and porcn-l.

Reduction in Porcn expression disrupts the convergent and extension movements in a cell non-autonomous manner

To precisely examine whether CE movement was impaired in porcn-deficient embryos, we analyzed the expression of several mesoderm markers. The expression of ntl was used to mark the notochord in tailbud-stage embryos. The notochord was apparently shorter along the anterior–posterior (A–P) axis and mediolaterally expanded in embryos injected with porcn MO than in the control embryos (Fig. 2H,I). In addition, broader and shorter paraxial mesoderm in the tailbud stage of porcn MO-injected embryos was confirmed by myoD (Fig. 2J,K,O,P) and papc (Fig. 2M,N) expression. By contrast, the expression of a dorsal marker, chd, and a ventral marker, bmp2b, in early gastrula were apparently normal in porcn-deficient embryos (Fig. 2R,S,U,V), indicating that the dorsal–ventral patterning in these embryos was unaffected. These expression patterns, as well as morphological abnormalities, are characteristic of mutants such as pipetail/wnt5b, silberblick/wnt11, knypek and trilobite, in which the CE movement is impaired (Westfall et al., 2003; Jessen et al., 2002; Topczewski et al., 2001).

We also observed an abnormality in cell intercalation during CE when analyzing the shape and orientation of notochord cells in porcn-deficient embryos at the four-somite stage (Fig. 3A–D). Cells in the control embryos were elongated having a length-to-width ratio (LWR) of 5.1±0.5 and aligned mediolaterally at an average angle of 4.8±2.4° relative to a line perpendicular to the A–P embryonic axis (Fig. 3A,C,D). By contrast, cells in the porcn-deficient embryos were rounder with an average LWR of 2.8±0.79 and aligned more randomly at a greater angle (11.9±3°) (Fig. 3B–D).

Furthermore, we examined movement of porcn-defective cells by transplantation of biotin-labeled mesoderm cells from control or porcn-defective donor embryos into the margin adjacent or lateral to the shield of control or porcn-defective host embryos (Fig. 3E–K). Whereas transplanted cells from control embryos moved anteriorly and formed strings of cells in their control hosts (Fig. 3G,J,L), porcn-defective cells moved in a more spreading fashion in porcn-defective hosts (Fig. 3H,K). Furthermore, porcn-defective cells moved in a similar manner to control cells when transplanted into control host (Fig. 3L,L). Thus, cells in porcn-defective embryos did not move properly during CE movement, and the porcn deficiency affected cells in a cell non-autonomous manner.

As well as loss of function, gain of function in components of the non-canonical Wnt pathway can cause abnormal CE phenotype (Kilian et al., 2003; Unger et al., 1995; Matsui et al., 2005); therefore, we examined the effect of overexpression of Porcn on CE movement. Microinjection of 75–150 pg porcn mRNA into wild-type embryos caused defects in axial elongation (Fig. 2G) and in gene expression (Fig. 2L,Q,T,W) similar to those of porcn morphants. Taken together, porcn appeared to be required for proper CE movement in a cell non-autonomous manner.

Genetic interaction of porcn with wnt5b and wnt11 in the CE phenotype

To determine whether porcn function was associated with Wnt function during CE movement, we examined genetic interactions between porcn and wnt5b (pipetail) and wnt11 (silberblick), both of which are known to be required for CE (Kilian et al., 2003; Heisenberg et al., 2000). Whereas injection of low concentrations of either the porcn MO or wnt5b MO led to no significant defects, co-injection of a low concentration of both morpholinos together yielded CE defects in 80% of the injected embryos (Fig. 4A–I), indicating that genetic interactions between these two genes could lead to the CE phenotype. Similarly, CE defects were observed when low concentrations of porcn MO and wnt11 MO were co-injected (Fig. 4J–R). Thus, we concluded that porcn plays a role in concert with wnt5b and wnt11 in mediating CE movements.

porcn-deficient embryos exhibit no obvious abnormality in developmental processes regulated by canonical Wnt signaling

Although porcn deficiency caused severe defect in CE movement, it did not result in the phenotypes caused by deficits in several other Wnt genes. For instance, whereas a deficit of wnt8 causes anteriorization of neural tissues, as indicated by expanded expression of six3, an eye primordial marker, and an anterior shift of the expression of eng2, a midbrain marker (Lekven et al., 2001; Kim et al., 2002), the expression of these marker genes was not altered in porcn-deficient embryos (Fig. 5A–C,G–I). Defects in the expression of
These marker genes were not observed even in embryos co-injected with either of additional porcn-specific MO (porcn MO') or porcn-1 MO with porcn MO (Fig. 5E,J,K). Furthermore, the porcn MO did not obviously enhance the phenotypes of wnt8, wnt3a, wnt1 or wnt10b morphants when porcn MO was co-injected with each wnt-specific MO (data not shown). Injection of porcn mRNA at a concentration that induced the CE defect did not cause any obvious abnormality in the expression of the above marker genes (Fig. 5F,L; data not shown).

Because wnt8, wnt3a, wnt1 and wnt10b induce canonical Wnt signaling in many cases, we examined targets of canonical Wnt signaling in porcn-deficient zebrafish embryos during epiboly, when porcn is ubiquitously expressed. Injection of porcn MO did not reduce the expression of the TOP–dGFP gene (Fig. 5M,N), a reporter of Wnt canonical signaling (Dorsky et al., 2002), nor that of axin2 (Fig. 5O,P), a target of the canonical Wnt signaling, in 80% epiboly embryos. Thus, in spite of the CE abnormality, developmental events regulated by canonical Wnt signaling appeared normal in porcn-deficient zebrafish embryos at least in early embryonic stages, suggesting that a deficit of porcn did not affect all the distinct events regulated by different Wnt proteins.

**Abnormal trafficking of Wnt5b, but not Wnt3a, from porcn-defective zebrafish embryonic cells**

One possible explanation for this non-equivalent effect of the porcn deficiency on different Wnt-mediated events is that the effects of this deficiency might be limited to specific cells or specific stages during development. Alternatively, this deficiency might not affect each member of the Wnt family in the same way. As a first step to assess these possibilities, we directly compared the effect of porcn depletion on the trafficking of several distinct Wnt molecules in their secretory process under the same set of conditions. Specifically, the trafficking of mouse and zebrafish Wnt3a, both of which can activate the canonical Wnt pathways in zebrafish embryos, was examined in epiblast cells of porcn-deficient zebrafish embryos and compared with the trafficking of several Wnt proteins, zebrafish Wnt5b and mouse Wnt5a, that are known to activate the non-canonical Wnt pathway. We injected mRNAs encoding EGFP-tagged forms of these Wnt proteins with porcn MO or a control MO into one-cell-stage embryos. Even with the addition of EGFP tags, Wnt proteins still retained biological activity, although this was reduced (Supplementary material Fig. S6). In embryos injected with the control MO, Wnt protein was distributed in cell-to-cell junction areas as puncta, indicating that the tagged proteins had been secreted or were in the process of secretion, as in the case of authentic Wnt3a observed in Xenopus embryos (Fig. 6A,C,E,G) (Takada et al., 2006). These puncta were not detected in un-injected embryos or when only secondary antibodies were used to probe the samples, indicating that the signals were specific (data not shown). Knockdown of porcn resulted in a change in the localization of zebrafish Wnt5b and mouse Wnt5a; most of these Wnt proteins
Fig. 4. *porcn* acts synergistically with *wnt5b* or *wnt11* in the regulation of CE movement. (A–I) Synergistic interaction of *porcn* with *wnt5b*. Live images of embryos injected with 1 ng of 5mis control *wnt5b* MO (A,C) or *wnt5b* MO (B,D) along with 0.5 ng of 5mis control *porcn* MO (A,B), or *porcn* MO (C,D). (E) The embryos shown in A–D were scored morphologically at 24 h.p.f. for the percentage of CE defects. Three independent experiments were scored and the percentage of normal, mildly or moderately CE defective embryos are indicated. n represents total number of embryos injected for each condition. Embryos injected with the same amounts of MOs in A–D were hybridized with *ntl* and *hgg1* probes are shown in F–I. (J–R) Synergistic interaction of *porcn* with *wnt11*. Embryos were injected with 1.5 ng of 5 mis control *wnt11* MO (J,L) or *wnt11* MO (K,M) along with 0.5 ng of 5mis control *porcn* MO (J,K), or *porcn* MO (L,M). The figures shown in J–R correspond to A–I, respectively. Combinatorial injection of suboptimal doses of *wnt5b* MO or *wnt11* MO and *porcn* MO resulted in a shorter and wider notochord.

Fig. 5. Canonical Wnt signaling is normal even in *porcn*-deficient and *porcn*-overexpressing embryos. (A–L) The anterior–posterior patterning of the brain examined by expression of *six3* (A–F) and *eng2* (G–L) in embryos injected with 1.5 ng of 5mis control MO (A,G), 2.5 ng each of two *wnt8* MOs (B,H) 1.5 ng of *porcn* MO (C,I), 1.5 ng of *porcn* MO and 5 ng *porcn* MO*"* (D,J), 1.5 ng *porcn* MO and 5 ng *porcn-l* MO (E,K), and with 100 pg *porcn* mRNA (F,L). All the embryos were stained at the one-somite stage. In double MO injection experiments (D,E,J,K), higher amounts of these MOs caused widespread cell death and embryonic death probably as a result of off-target effects of these MOs. (M,N) TOP–dGFP transgenic embryos were injected with 1.5 ng of 5mis control MO (M) or *porcn* MO (N) and were stained with the gfp probe at the 80% epiboly stage. (O,P) Embryos were injected with 1.5 ng of 5mis control MO (O) or *porcn* MO (P) and were stained with the axin2 probe at the 80% epiboly stage.
were not localized to cell-to-cell junctions, but mainly found within the cytoplasm (Fig. 6B,D). In some cells that expressed more zebrafish Wnt5b, the cytoplasmic signals were condensed as puncta (data not shown). By contrast, neither zebrafish nor mouse Wnt3a distribution was changed by depletion of porcn (Fig. 6F,H). These results indicate that porcn deficiency affected the trafficking of mouse Wnt5a and zebrafish Wnt5b proteins, but not mouse and zebrafish Wnt3a, in zebrafish epiblast cells.

**Deficiency of Porcupine does not equivalently affect lipidation of different Wnt proteins in zebrafish embryos**

The result showing that Wnt3a was normally secreted even in porcn-deficient zebrafish embryos led us to speculate that Wnt3a might not require lipidation for its secretion in zebrafish embryos. However, a mutant form of mouse Wnt3a lacking the lipidation (palmitoleoylation) site, was not trafficked into the cell-to-cell junctions but remained in the cells, as in the case of mouse mutant Wnt5a (supplementary material Fig. S7). Thus, contrary to our speculation, mouse Wnt3a seems to require lipidation for its secretion in zebrafish embryos.

These results strongly suggest that Wnt3a, but not zebrafish Wnt5b and mouse Wnt5a, is normally lipidated even in porcn-deficient zebrafish embryos. To test this possibility more directly, we compared the hydrophobicity of zebrafish Wnt3a and Wnt5b and mouse Wnt5a in porcn-deficient embryos with that in control embryos by temperature-induced phase separation in Triton X-114 (Fig. 7). First, we confirmed that decrease of the hydrophobicity of a lipidation-defective form of mouse Wnt3a, in which Ser209 was substituted with Ala, was actually detected in this assay (Fig. 7A). In embryos injected with control MO, all Wnt proteins were partitioned into both the aqueous and detergent phases, probably reflecting the pre-palmitoleoylated and the palmitoleoylated forms, respectively, although the ratio of amounts partitioned into the two phases were different among the three Wnt proteins (Fig. 7B–D). Whereas the relative proportion of zebrafish Wnt5b, as well as mouse Wnt5a, in the aqueous fraction was apparently increased in embryos injected with porcn MO (Fig. 7C,D), that of zebrafish Wnt3a was apparently unchanged. Thus, the porcn deficiency increased the unlipidated population of zebrafish Wnt5a and mouse Wnt5b, but not that of Wnt3a.

**Secretion of Wnt5a is inhibited by less Porcn inhibitor than that of Wnt3a in HEK293T cells**

These results showed that porcn is preferentially required for trafficking and lipidation of a particular Wnt, Wnt5b, in early zebrafish embryos. However, this appeared to be inconsistent with our previous results in mouse L cells, in which the porcn deficiency affected the secretion of both Wnt5a and Wnt3a. Therefore, we performed more precise analyses to examine the effect of porcn deficiency in mammalian culture cells by depressing the level of Porcn activity with a Porcn-specific inhibitor, IWP-2. HEK293T cells were transiently transfected with mouse Wnt3a or Wnt5a and treated with different concentrations of IWP-2 under the same conditions. Whereas only 10–20 nM IWP-2 was effective for 50% inhibition of mouse Wnt5a secretion, mouse Wnt3a required high doses of around 100–200 nM IWP-2. Thus, the decrease of Porcn activity does not equivalently affect the secretion of different Wnt proteins in culture cells, as in the case of their trafficking in early zebrafish embryos.

**Discussion**

In this study, we unexpectedly found that zebrafish embryos defective in porcn exhibited only limited phenotypes caused by deficiency of Wnt signaling. Whereas the CE movements were defective in porcn-deficient embryos, these embryos did not show any defects specifically caused by a reduction in canonical Wnt signaling. Consistent with this result, neither expression of axin2, an endogenous target of canonical Wnt signaling, nor that
of TOP-dGFP, a reporter of canonical signaling, was reduced in porcn-deficient embryos. Thus, the porcn MO phenotype looked strikingly similar to those phenotypes of pipetall/wnt5b, silberblick/wnt11, knypek and trilobite, in which the CE movement is specifically impaired.

To gain more insight into the reason why CE movement was specifically impaired in porcn-deficient embryos, we directly compared trafficking and lipidation of different Wnts in porcn-deficient embryonic cells. A precise comparison of localization of ectopically expressed Wnt proteins under the same conditions showed that the trafficking of zebrafish Wnt5b and mouse Wnt5a, but not zebrafish and mouse Wnt3a, was impaired by a reduction in porcn. Although Wnt3a trafficking was unaffected in porcn-deficient embryos, our results suggested that lipidation was still required for Wnt3a trafficking in zebrafish embryos because a Wnt3a mutant that lacked the palmitoleoylation site was retained within the cells. If this holds true, lipidation of Wnt3a should be unaffected by injection of porcn MO. Consistent with this, we found that lipidation of Wnt3a was similar between normal and porcn-deficient embryos. By contrast, lipidation of zebrafish Wnt5b and mouse Wnt5a was reduced in porcn-deficient embryos. Thus, porcn deficiency caused by injection of porcn-specific MOs did not equivalently affect all Wnt proteins as examined in epiblast cells in zebrafish embryos. Similarly, differences in the effect of porcn deficiency on Wnt5a and Wnt3a secretion were observed in HEK293 cells. These results suggest that the non-equivalent effect of Porcn on several different Wnt proteins is the reason for the generation of the CE limited phenotype of porcn-defective embryos.

There are several possibilities to explain how porcn deficiency non-equivalently affected the trafficking and lipidation of different Wnt proteins in zebrafish embryos. Given that the porcn morphant is not null but hypomorphic, one possible explanation is that residual amounts of Porcn in the porcn morphants were sufficient for several Wnt proteins including Wnt3a, whereas some particular Wnts require less Porcn for their lipidation. Consistent with this explanation, we found mouse Wnt5a required less Porcn activity for its secretion than Wnt3a in HEK293T cells. Another possibility is that some other acyltransferase might specifically catalyze particular types of Wnt proteins, including Wnt3a. Possible candidates for such an enzyme are members of the MBOAT family of membrane-bound acyltransferases. In this study, we examined whether a member of this family, Porcn-l, could have a role in the regulation of Wnt signaling by injecting a specific MO. However, our results did not support this possibility up to now.

Interestingly, we observed that porcn was not ubiquitously expressed after the mid-somite stage in zebrafish embryos. Such non-ubiquitous expression of porcn is also observed in mouse embryos (Biechele et al., 2011). Of note, in the neural tube of zebrafish embryos, porcn expression was detected in the ventral side, but not in the dorsal side, where several wnt genes, including wnt3a are expressed. This expression pattern of porcn suggests that some unidentified machinery, including another acyltransferase might be involved in Wnt secretion in the dorsal neural tube of zebrafish embryos. More extensive analyses in the future will reveal the precise mechanism of Wnt lipidation in vertebrate embryos.

In conclusion, we found that trafficking and lipidation of at least some Wnt ligands, such as zebrafish Wnt5b and mouse Wnt5a, required Porcn activity in early zebrafish, whereas trafficking and/or lipidation of other Wnt ligands, zebrafish and mouse Wnt3a, required less or no Porcn activity. Similarly, secretion of mouse Wnt5a required more Porcn activity than that of Wnt3a in mammalian culture cells. This is the first report to show that a decrease of Porcn does not equivalently affect trafficking and lipidation of different Wnt proteins. Our findings indicate that the machinery required for Wnt protein secretion is not as simple as previously predicted; rather, secretion of distinct Wnt proteins appears to be differentially regulated.
Materials and Methods

Zebrafish maintenance
Zebrafish of the TL and India strains were maintained and bred according to standard procedures (Westerfield, 2000).

Cloning of zebrafish porcn cDNAs
By a BLAST analysis with the 9th zebrafish genome assembly released from the Sanger Institute, we found two genes, porcn and porcn-l, to be related to porcn genes identified in other species. mRNA was harvested from embryos 24 hours after fertilization and cDNA was synthesized by reverse transcription using random primers. This cDNA was used to amplify the porcn genes by PCR using the specific primers described in supplementary material Table S1A. Both cDNAs encoded the same type of splicing variants corresponding to PorcnD in mouse (Tanaka et al., 2000; Caricasole et al., 2002). A phylogenetic analysis and tree construction of porcupine sequences were conducted by using CLUSTALW (http://www.ebi.ac.uk/Tools/es/cgi-bin/clustalw2).

RNA and morpholino antisense oligonucleotide (MO) injection
Capped sense RNAs encoding the full-length mouse Porcn, Myc-tagged zebrafish Porcn, m-Venus (Wallingford et al., 2000), truncated forms of zebrafish porcn and porcn-l tagged with EGFP, FLAG- and EGFP-tagged zebrafish wnt5b, zebrafish wnt3a, mouse Wnt3a, and mouse Wnt5a were synthesized using mMessage Machine system (Ambion). All injections were performed on one-cell stage embryos. For the sequences of MOs, see supplementary material Table S1B.

Cell transplantation
For transplantations, donor embryos were injected with a mixture of Rhodamine and biotin–dextran plus either 1.5 ng of porcn MO or 1.5 ng of 5mis control MO at the one-cell stage. Groups of donor cells were taken from shield-stage embryos and transplanted into the deep region (close to the embryonic shield) or into the lateral germ ring of host embryos that were injected with either 5 mis control or porcn MO as described previously (Heisenberg et al., 2000). Host embryos were harvested at the one-somite stage and fixed for in situ hybridization. Transplanted cells were detected with a Vectastain Kit (Vector Laboratories) to reveal the biotin labeling.

In situ hybridization and notochord cell shape analysis
Whole-mount in situ hybridization was carried out as previously described (Westerfield, 2000). Antisense RNA probes were synthesized with digoxigenin RNA labeling kits (Roche Diagnostics). To investigate the shapes of notochord cells, embryos were injected with 20 pg of membrane-bound Venus mRNA and 1.5 ng of 5mis control or porcn MO and were then fixed with 4% paraformaldehyde (PFA) at the four-somite stage. Immunostaining of mVenus was performed as described previously (Westerfield, 2000). The antibody used for detection was 598 anti-GFP (1:3000, MBL). Length-width-ratio (LWR) of notochord cells and the angles of the long axis relative to a line perpendicular to the embryonic axis as represented by the notochord were analyzed using Object-image software.

Analysis of subcellular protein localization
Wnt proteins tagged with FLAG and EGFP (EGFP–Wnts) had the signal peptide of mouse Wnt3a added before these tags. Embryos at the one-cell stage were co-injected with a RNA encoding EGFP–Wnt (6.25 pg of zebrafish wnt3a, mouse Wnt3a or mouse Wnt5a mRNA was used, but 12.5 pg of zebrafish wnt3b mRNA was used) and 15 pg of membrane-bound RFP with either 2.5 ng of 5mis control MO or porcn MO. Embryos were fixed at 80–90% epiboly and anti-GFP, anti-RFP or anti-β-catenin immunostaining was performed as previously described (Westerfield, 2000). Images were acquired using a confocal microscope. The following antibodies were used: 598 anti-GFP (1:3000, MBL), M155-3 anti-RFP (1:2000, MBL), #C19200 anti-β-catenin (1:500, Transduction Labs), anti-rabbit IgG antibody conjugated to Alexa Fluor 488 (1:500, Molecular Probes), and anti-mouse IgG antibody conjugated to Alexa Fluor 546 (1:500, Molecular Probes).

Cell culture, transfection and western blotting
For mouse Porcn-knockdown cells, pSilencer 3.0-H1 plasmid, expressing an siRNA specific for Porcn, was used to transfect cells that stably expressed and secreted Wnts level relative to that of cell lysate was calculated and presented as percentage of the control. Results are means ± s.e.m.

Fig. 8. Secretion of mouse Wnt5a is more susceptible than Wnt3a to Porcupine inhibitor IWP-2.
(A,B) HEK293T cells transiently expressing other mouse Wnt3a or Wnt5a were treated with IWP-2 compound at different concentrations as indicated for 48 hours before lysis. Cell culture medium were subjected to western blot analysis to determine levels of secreted Wnts. Standards to enable quantification of secreted Wnts were also loaded (left). The level of Wnt protein in cell lysate was not obviously changed by treatment with IWP-2.
(C) Quantification of secreted Wnts in conditioned medium. Western blot images were scanned and Wnt protein bands were quantified using ImageJ software. For each lane, the secreted Wnts level relative to that of cell lysate was calculated and presented as percentage of the control. Results are means ± s.e.m.
secreted Wnt3a or Wnt5a as previously described (Takada et al., 2006). Stable clones with varying levels of defective mouse Porcn expression were confirmed by real-time RT-PCR.

Mouse L cells deficient in Porcn expression but stably expressing either Wnt3a or Wnt5a were plated at density of 2 × 10^6 cells/cm^2 and transiently transfected with Myc-tagged porcn, porcn-1 or control vector as indicated. Culture supernatants or cell lysates were collected 36 hours after transfection. The final loading amount of each sample on the SDS-PAGE gel was normalized to cell number calculated at the harvest time point. Proteins on the SDS-PAGE gel were blotted to a PVDF membrane and probed with anti-Myc (1:2000, MBL), anti-Wnt3a or anti-Wnt5a monoclonal antibody as described previously (Takada et al., 2006).

Triton X-114 phase separation
Twenty-five pg of the wild-type or a palmitoleoylation-defective (S209A) form (Takada et al., 2006) of EGFFP-tagged mouse Wnt1a mRNA was injected into zebrafish embryos at the one-cell stage. Twenty five pg of EGFFP-tagged zebrafish wnt3a, wnt5b or mouse Wnt3a, and either 2.5 ng or 50 μg control MO were also injected as indicated. 100 injected embryos were lysed in 500 μl of ice-cold Triton X-114 lysis buffer (100 μl of Tris-buffered saline-condensed Triton T114-400 and 400 μl of Tris-buffered saline containing protease inhibitors) and homogenized by passing through a 27-gauge syringe 20 times. The embryo lysate was then centrifuged at 4°C at 1000 g for 8 minutes and then centrifuged at 1000 g at room temperature for 8 minutes. The aqueous phase was transferred to a new tube and mixed with 0.5% fresh Triton X-114. The surfactant was dispersed on ice for 10 minutes and the mixture was again overlaid on the sucrose cushion used previously. This mixture was then incubated at 30°C for 3 minutes at 1000 g at room temperature for 3 minutes. After separation, Triton X-114 and Tritis buffer were added to the aqueous and detergent phase, respectively, to obtain approximately the same salt and surfactant content in both samples. The aqueous phase and the detergent phase were subjected to 8% SDS-PAGE and analyzed by western blotting.

Porcine inhibitor (IWP-2) treatment and Western blot image analysis
To HEK293T cells were transiently transfected with mouse Wnt3a or Wnt5a cDNA using GeneJuice transfection reagent (Merck4Bioscience). The medium was replaced with fresh DMEM 5 hours after transfection and supplemented with 0–1000 nM IWP-2 as previously described (Chen et al., 2009). IWP-2 was re-replaced with fresh DMEM 5 hours after transfection and supplemented with using GeneJuice transfection reagent (Merck4Bioscience). The medium was

Acknowledgements
We thank thank S. Koshida, T. Akanuma, T. Yabe, H. Takahashi, and Ms. H. Utsumi for their technical support, R. Moon and T. Buckle.

Funding
This work was supported in part by a grant-in-aid for scientific research (to S.T.) from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

Supplementary material available online at http://jcs.biologists.orglookup/sumpli.doi:10.1242/jcs.098368/-/DC1

References


Becich, M., Kishida, T., Ak, Y., Yabe, H. Takahashi, and Ms. H. Utsumi for their technical support, R. Moon and T. Buckle.


Table S1

(A) A list of primer sets for RT-PCR to clone zebrafish *porcn* genes and identify *porcn* splicing variance

<table>
<thead>
<tr>
<th>Gene</th>
<th>primer set</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>porcn</em></td>
<td>5’-gttcttcagtgcaacagtg-3’ and 5’-tctategccttacctaag-3’</td>
</tr>
<tr>
<td><em>porcn-l</em></td>
<td>5’-gacctggtgcgtgctgagat-3’ and 5’-atcaggttcagcgaatggc-3’</td>
</tr>
<tr>
<td>Splicing variance</td>
<td>5’-cagctctgcctctcatttc-3’ and 5’-gtggtggtgtgctcagcgg-3’</td>
</tr>
</tbody>
</table>

(B) Morpholinos (MO) used in this study

<table>
<thead>
<tr>
<th>Morpholino name (MO)</th>
<th>sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>porcn</em> MO</td>
<td>5’-GCCTGTCTGCTCAGAGATCCCATTTG-3’</td>
</tr>
<tr>
<td><em>porcn</em> MO’</td>
<td>5’-TTTCACCTGTTTAGACATAGAAAGAA-3’</td>
</tr>
<tr>
<td><em>porcn</em> MO”</td>
<td>5’-GGGATAATTCACACACAACACTCTTCC-3’</td>
</tr>
<tr>
<td>5mis <em>porcn</em> MO</td>
<td>5’-GCGTGTCTCCTCAAGATCCGATAGAGG-3’</td>
</tr>
<tr>
<td>5mis <em>porcn</em> MO’</td>
<td>5’-TTTCAGTCCTGAGACACATAGAAAGAA-3’</td>
</tr>
<tr>
<td><em>porcn-l</em> MO</td>
<td>5’-CCATACGCCCAGAAGTCCCATAG-3’</td>
</tr>
<tr>
<td><em>porcn-l</em> MO’</td>
<td>5’-TCACCTAGCCCATACAGAGTCCCATAGG-3’</td>
</tr>
<tr>
<td>5mis <em>porcn-l</em> MO</td>
<td>5’-TCgATACcCCCATAcGAATCAGCAG-3’</td>
</tr>
</tbody>
</table>

The *wnt1*, *wnt3a*, *wnt5b*, *wnt8*, *wnt10b*, *wnt11*, and *p53* MO sequences were designed as previously described (Lekken et al., 2003; Buckles et al., 2004; Kilian et al., 2003; Lekken et al., 2001; Zhu et al., 2006; Robu et al., 2007).