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Porcupine homolog is required for canonical Wnt signaling and gastrulation in mouse embryos

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ABSTRACT

Wnt signaling plays important roles in development and disease. The X-chromosomal *Porcupine homolog* gene (*Porcn*) encodes an evolutionary conserved member of the membrane bound O-acyl transferase (MBOAT) superfamily that has been shown to be required for the palmitoylation and secretion of Wnt3a, a mechanism that has been suggested to be conserved for all mammalian Wnt ligands. *PORCN* mutations in humans cause Focal Dermal Hypoplasia (FDH), a disorder causing developmental defects in heterozygous females and embryonic lethality in hemizygous males. In this study, *Porcn* mutant mouse embryonic stem (ES) cells were used to analyze the role of *Porcn* in mammalian embryonic development. *In vitro*, we show an exclusive requirement for *Porcn* in Wnt secreting cells and further, that any of the four *Porcn* isoforms is sufficient to allow for the secretion of functional Wnt3a. Embryos generated by aggregation of *Porcn* mutant ES cells with wildtype embryos fail to complete gastrulation *in vivo*, but remain in an epiblast-like state, similar to *Wnt3* and *Gpr177/Wls* mutants. Consistent with this phenotype, *in vitro* differentiated mutant ES cells fail to generate endoderm and mesoderm derivatives. Taken together, these data confirm the importance of *Porcn* for Wnt secretion and gastrulation and suggest that disruption of early development underlies the male lethality of human *PORCN* mutants.

Introduction

Focal Dermal Hypoplasia (FDH, Goltz Syndrome) (OMIM ID: 305600) is an X-linked dominant disorder that is characterized by dysplasias in ecto-mesodermal tissues. Mutations in the human *Porcupine homolog* (*Porcn*) gene on the X chromosome (Xp11.23) have been identified as the cause for FDH (Grzeschik et al., 2007; Wang et al., 2007). The majority of FDH patients are females, and all male patients carry postzygotic mutations, making them mosaic for *Porcn* function. The absence of zygotic mutant male patients indicates likely embryonic lethality caused by complete lack of *Porcn* function in humans (Bornholdt et al., 2009). We have recently identified *Porcn* in a screen for X-chromosomal embryonic lethal genes in the mouse (Cox et al., 2010), providing evidence that *Porcn* is indeed required for mammalian embryonic development. Molecularly, *Porcn* is a member of the membrane bound O-acyl transferase (MBOAT) superfamily (Hofmann, 2000) and is proposed to act as a key lipid modifier of Wnt ligands (Chen et al., 2009; Takada et al., 2006).

In mammals, Wnt ligands act through multiple pathways, including the canonical Wnt signaling pathway, which activates transcription of target genes through beta-catenin/TCF/LEF complexes, and the non-canonical planar cell polarity (PCP) pathway, which is involved in polarization of cells within an epithelial sheet and during convergent extension movements (MacDonald et al., 2009). Wnt proteins are signaling molecules with important roles in embryonic development as well as in tissue homeostasis and stem cell maintenance after birth (Clevers, 2006; Logan and Nusse, 2004). Consistent with these functions, mutations or deregulation of Wnt pathway components have been associated with multiple developmental defects as well as cancer (Niemann et al., 2004; Polakis, 2007).

While most studies have been directed towards identifying and characterizing the mechanisms acting in the Wnt receiving cells, there is growing interest in the processes required for post-translational modification and Wnt secretion (Port and Basler, 2010). Wnt proteins have a size of approximately 40 kDa and contain a number of highly conserved cysteine residues. While the amino acid composition of Wnts suggests a polar, water-soluble behavior, their actual hydrophobic behavior has been attributed to two lipid-modifications; palmitoylation of the N-terminal cysteine (C77 in mouse Wnt3a, (Willert et al., 2003)) and modification of a conserved serine residue (S209) with mono-unsaturated palmitoleic acid (Takada et al., 2006). Post-translational lipid-modification has also been confirmed biochemically for Wnt1 (Galli et al., 2007) and Wnt5a (Kurayoshi et al., 2007) and is thus conserved in all Wnts examined to date, with the exception of Drosophila WntD (Ching et al., 2008). WntD does not

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carry any lipid-adducts (Ching et al., 2008) and also lacks the two lipid modified residues that are conserved in all mammalian Wnts. It is therefore predicted that lipid modification is a common feature of Wnt ligand processing. Porcn has been shown to be required for the palmitoylation of several Wnt ligands (Wnt3a (Takada et al., 2006), Wnt1, Wnt5a (Chen et al., 2009)) and interacts with several other Wnts in over-expression studies (Tanaka et al., 2000). Importantly, loss of function studies in Drosophila (Kadowaki et al., 1996; Zhai et al., 2004) and overexpression and dominant negative approaches in chick (Galli et al., 2007) all indicate that *Porcn*-mediated lipid-modifications are required for proper processing of Wnt ligands in the ER, and for their extracellular distribution and gradient function.

While RNAi knockdown (Takada et al., 2006), chemical inhibition (Chen et al., 2009) and dominant negative (Galli et al., 2007) approaches have all provided valuable insights into *Porcn* function and the associated Wnt gradient formation, a genetic *Porcn* null vertebrate model organism has not been described to date. Using *Porcn* null embryonic stem (ES) cells, we show that *Porcn* is required for generation of endoderm and mesoderm in embryoid bodies *in vitro* and in Wnt expressing ES cells for secretion of functional Wnt3a. *In vivo*, *Porcn* is required for correct gastrulation and germ layer establishment. In *Porcn* mutants epiblast marker genes are not downregulated and mesoderm and endoderm differentiation is disrupted. We suggest that this major developmental disruption underlies the embryonic lethality of human *Porcn* mutant male embryos.

Material and methods

ES cell culture

E14Tg2a.4 (parental wildtype) and CSD256 (*Porcn* genetrap) cells were obtained from Baygenomics and cultured in feeder-free, serum-free conditions containing 1000 U/ml Leukemia Inhibitory Factor (LIF), 1 μM PD0325901 (Mek inhibitor, Stemgent) and 3 μM CHIR99021 (Gsk3 inhibitor, Stemgent) as previously described (Nichols and Ying, 2006). Tissue culture dishes were coated with gelatin and ES-qualified fetal calf serum (Hyclone) immediately before plating of ES cells.

Constructs

For over-expression experiments, pCX-EYFP (Hadjantonakis et al., 2002) was used as a control plasmid for mock transfections. The promoter/enhancer elements in this vector drive strong, widespread transgene expression in ES cells and live mice (Niwa et al., 1991). The *Porcn*C ORF was amplified by PCR from a Mammalian Gene Collection (MGC) clone (accession: BC032284) whereas Wnt3a and *Porcn* isoforms A, B and D from previously published vectors (gift from T. Kadowaki) (Tanaka et al., 2000). Wnt3a point mutants C77A, S209A, and C77AS209A were generated by site-directed mutagenesis of the wild-type Wnt3a ORF. Amplicons were ligated into the EcoRI site of pCX-EYFP to replace the EYFP ORF. All plasmids were verified by sequencing.

Canonical Wnt activity assay

For the initial autocrine assay, ES cells were plated at 200,000 cells/well in 24 well plates in serum-free ES media containing LIF, but no PD0325901 or CHIR99021. Cells were transfected in triplicate at time of plating with 20 ng pCMV-RenillaLuciferase (pCMV-RL), 400 ng Super8-XTOPFlash (or Super8XFOPFlash) (Veeman et al., 2003), as well as pCX-EYFP (500 ng/1000 ng), pCX-Wnt3a (500 ng), pCX-PorcnA-D (total amount: 500 ng) using FugeneHD (Roche). Luciferase activity was determined 24 h after transfection using Dual-Luciferase® Reporter Assay System (Promega) according to manufacturer's protocol.

The autocrine Wnt3a point-mutant assay was performed as above, with the following modification; 500 ng of pCX-Wnt3a (wt or point-mutants) or 500 ng of pCX-EYFP were transfected along with 20 ng of pCMV-RL and 400 ng Super8XTOPFlash (or Super8XFOPFlash).

For paracrine assays, cells were transfected with pCX-Wnt3a or pCX-EYFP using FugeneHD in serum-free media without inhibitors. Recipient cells were transfected with pCMV-RL and Super8XTOPFlash (or Super8XFOPFlash). 24 h after transfection cells were dissociated using Trypsin and 75,000 producer cells and 75,000 recipient cells were plated per well of a 24 well plate in triplicate. Luciferase activity was determined 24 h after cell mixing using Dual-Luciferase® Reporter Assay System (Promega) according to manufacturer's protocol.

All Luciferase assays were performed at least three times with three technical replicates. Results were analyzed using Student's *t*-test.

Nodal activity assay

Assay was performed and analyzed as described for the autocrine canonical Wnt signaling assay with the following modifications: Inhibitors PD0325901 and CHIR99021 were included in the media. Cells were transfected with 500 ng pBOS-Nodal (Yamamoto et al., 2003) (or pCX-EYFP (Hadjantonakis et al., 2002)), 300 ng $(n2)_7$-luc (Saijoh et al., 2000) (gift from H. Hamada) and 20 ng pCMV-RL. For inhibition and activation of the pathway, 10 μM SB431542 (Sigma) and 100 ng/ml Activin A (R&D Systems) were used respectively.

Embryo generation

Porcn null embryos were generated by aggregation of *Porcn* genetrap ES cells (CSD256, Baygenomics) with 8-cell stage embryos as previously described (Cox et al., 2010). Briefly, male mice homozygous for a ubiquitously expressed eGFP transgene (B5/EGFP) [Tg(CAG-EGFP)B5Nagy] (Hadjantonakis et al., 1998) were mated with superovulated ICR (Harlan) female mice and embryos were collected at day E1.5. After over-night culture until the uncompacted eight-cell stage, each embryo was aggregated with a clump of 8 to 15 ES cells in depression wells and cultured one more night, before morulae and blastocysts were transferred into the uteri of pseudopregnant females at E2.5. Embryos were dissected five (E7.5) or six days (E8.5) after embryo transfer and fixed in 4% PFA in PBS overnight.

Whole-mount in situ hybridization

Whole-mount embryo *in situ* hybridization was performed as previously described (Yamanaka et al., 2007). The RNA probes used were: *Axin2* (HindIII fragment, F. Costantini), *Brachyury* (Herrmann, 1991), *Cer1* (Shawlot et al., 1998), Hesx1 (Thomas and Beddington, 1996), *Hoxb1* (Wilkinson et al., 1989), *Lhx1* (Shawlot and Behringer, 1995), *Otx2* (Ang et al., 1994), *Wnt3* (Roelink et al., 1990). For *in situ* hybridization of *Porcn*, two probes were used simultaneously. The templates for *Porcn* probes were amplified from cDNA clones by PCR using primers Fwd1: CTGGAATGGGACCTGACAGT, Rev1: GCATC-CAAAAGTGACCCAGT, Fwd2: GTGAGATGCACATGGTGGAC, Rev2: ACTGTCAGGTCCCATTCCAG and cloned into pBSII for transcription with sp6/T7 polymerases.

In vitro differentiation and flow cytometric analysis

Serum-free *in vitro* differentiation as embryoid bodies (EB) was performed as previously described (Gadue et al., 2006) with minor modifications; ES cells were cultured in differentiation media at 75,000 cells/ml on low attachment plates (Costar). For standard differentiation, 5 ng/ml Activin A (R&D), 0.5 ng/ml rhBmp4 (R&D) were added after 2 days and EBs were cultured for another 2 days without dissociation/re-aggregation. Other factors used: 1 μM IWP-2 (*Porcn* inhibitor, gift from L. Lum); 3 μM CHIR99021 (Stemgent), 150 ng/ml rmDkk (R&D), 20–200 ng/ml rmWnt3a (R&D).

For endoderm differentiation, 100 ng/ml Activin A (R&D) was added at day 2 without addition of Bmp4.

For flow-cytometric analysis, EBs were dissociated by incubation with Trypsin/EDTA and stained using the following antibodies: Phycoerythrin (PE) Rat anti-mouse Flk-1 (BD Pharmingen), Allophycocyanin (APC) Rat anti-Mouse CD184 (CXCR4, BD Pharmingen) and fluorescein isothiocyanate (FITC) Mouse anti-SSEA-1 (BD Pharmingen). The cell suspension was analyzed on a LSRII or FACSCanto flow cytometer (BD Biosciences) and dead cells were excluded from the analysis based on propidium iodide staining. Data analysis was performed using FlowJo Software (Tree Star Inc.).

Quantitative Real-time PCR analysis

RNA was isolated (Trizol Reagent, GIBCO BRL) at day 0, 2 and 4 of EB differentiation according to the manufacturer's instructions. Two micrograms of RNA were reverse transcribed using QuantiTect RT Kit (Qiagen) according to manufacturer's protocol. Generated cDNA was used for gene expression analysis by Real-time PCR on a Roche LightCycler 480 using 2X SYBR master mix (Roche). Primers and PCR conditions see Supplementary Table S1. Melt curve analysis was used to determine primer specificity and standard curves were generated to control for primer efficiency. Gene expression was determined by relative quantification with values corrected for input using GAPDH and normalized relative to wildtype EBs at the respective time-point. Data presented in Fig. 6 was obtained in three independent experiments and analyzed using Student's *t*-test ($p<0.05$).

RT-PCR

RNA was extracted from E14Tg2a.4 and CSD256 ES cells using Trizol according to manufacturer's instructions. RNA was transcribed into cDNA using SuperScript™ III Reverse Transcriptase (Invitrogen) with oligo(dT) primers. Gene specific PCR was performed using Taq DNA Polymerase (Roche) with the following *Porcn*-Fwd (GTGAGATGCACATGGTGGAC) and *Porcn*-Rev (ACTGTCAGGTCCCATTCCAG) primers, spanning exons 4 to 9. Detection of H2afz transcript was used as control (Mamo et al., 2007).

Results

Porcn expression analysis in the peri-gastrulation mouse embryo

The 19 Wnt ligands encoded in the mouse genome are expressed dynamically both temporally and spatially throughout embryonic development. In order to determine whether *Porcn*, an MBOAT family member required for the acylation of several, possibly all, Wnt ligands, would have dynamic or ubiquitous expression during embryo development, we analyzed the expression of *Porcn* in the gastrulating mouse embryo using RNA *in situ* hybridization.

At E6.5, *Porcn* is expressed in epiblast cells undergoing gastrulation movements and forming the primitive streak on the posterior side of the embryo, as well as the AVE (Fig. 1A). One day later, *Porcn* is no longer expressed in the AVE but in the lateral proximal region of the endoderm layer surrounding the egg cylinder, the lateral regions of the epiblast and at lower levels in the primitive streak region and the migrating mesoderm (Fig. 1B and E). *Porcn* is not detectable in the anterior most region of the embryo (Fig. 1E). At E8.25, *Porcn* expression is restricted to the embryo proper with the exception of the region of the cardiogenic plate and foregut pocket (Fig. 1C). After turning (E9.0), *Porcn* is expressed strongly on the dorsal side of the embryo in the neural tube where it forms a gradient with highest expression levels on the dorsal side (Fig. 1D and G). Modest *Porcn* expression was detected throughout the cranial region and in the optic vesicles (Fig. 1D and F) with a marked absence of expression in the surface ectoderm (Fig. 1F). *Porcn* was also undetectable in the developing heart (Fig. 1D and G).

Although not ubiquitous, the expression pattern of *Porcn* at E9.0 is broad enough at these stages to encompass the published expression domains of the Wnt ligands (Witte et al., 2009). At gastrulation, *Porcn* expression on the posterior side of the gastrulating embryo is consistent with the expression pattern of several Wnt proteins, but its expression in the AVE was unexpected as this is a source of Wnt inhibitors (Kemp et al., 2005). *Wnt3* however, has been reported to be expressed in the visceral endoderm, proximal to the AVE (Rivera-Pérez and Magnuson, 2005). A functional relevance for this expression has not been reported to date. While it is possible that *Porcn* expression in the AVE has no function, it opens the possibility that *Porcn* may have non-Wnt substrates.

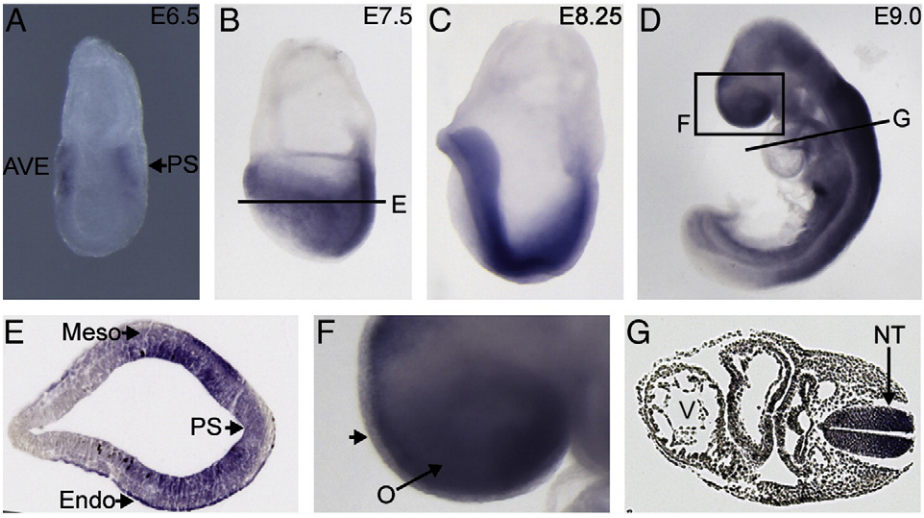

Fig. 1. *Porcn* expression pattern in peri-gastrulation embryos. *Porcn* expression was visualized in wildtype embryos by whole mount RNA *in situ* hybridization (A-D, F) and sections thereof (E, G). Gastrulation stage embryos and sections are oriented with their anterior to the left (A-C, E). (A) At 6.5, *Porcn* expression can be detected in the primitive streak (PS), as well as the anterior visceral endoderm (AVE). By E7.5 *Porcn* expression can be detected at moderate levels in the primitive streak (PS) and migrating mesoderm (Meso) and at higher levels in the lateral regions of the epiblast and the endodermal cell layer (Endo), but is still restricted to the embryo proper. At E8.25 (C), *Porcn* expression restricted to the embryo proper with the exception of the region of the cardiogenic plate and foregut pocket. After turning (D), *Porcn* is highly expressed in the neural tube (NT) and at moderate levels throughout the cranial region. *Porcn* expression cannot be detected in the surface ectoderm (arrow) of the head (F) overlying the optic vesicle (O). *Porcn* is also absent from the developing cardiac ventricle (V, Figures D, G).

Porcn genetrap ES cells exhibit defects in canonical Wnt signaling

We obtained a mouse male embryonic stem cell line (CSD256) from Baygenomics, carrying a genetrap insertion in the second intron of *Porcn*, 3′ to the open reading frame start site (Fig. 2A). This integration site is predicted to terminate *Porcn* transcripts after 136 bases of coding sequence, affecting all 4 isoforms that are generated by alternative splicing of exon 7 (18 bp) and exon 8 (15 bp) (Fig. 2A) (Tanaka et al., 2000). In contrast to cell lines carrying heterozygous genetrap insertions in autosomal genes, this cell line is a functional *Porcn* null cell line due to male hemizygosity. Absence of *Porcn* transcripts was confirmed by RT-PCR using exon-junction spanning primers that allow detection of all 4 isoforms (Fig. 2B).

As *Porcn* has been shown to be involved in the lipid-modification of Wnt ligands in both mammals and flies, we tested whether *Porcn* was required for canonical Wnt signaling using Tcf/Lef-luciferase

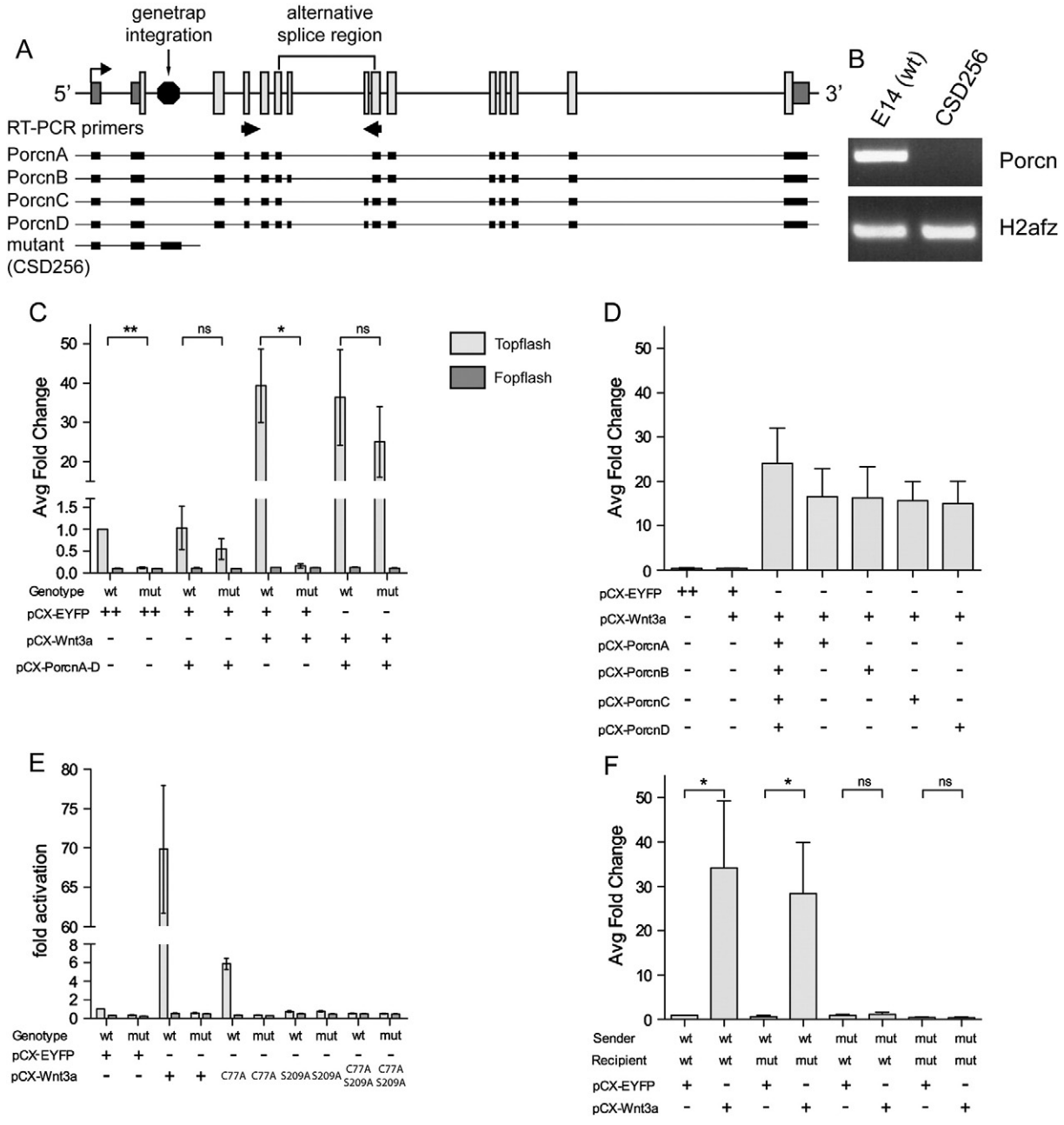

Fig. 2. Validation of *Porcn* genetrap ES cell line and canonical Wnt signaling defects. (A) Schematic representation of *Porcn* locus with genetrap integration site and *Porcn* transcripts. Untranslated regions of *Porcn* are indicated in dark gray, translated regions in light gray. Primers for RT-PCR are indicated by arrows and allow amplification of exons 4–9. Wildtype *Porcn* transcripts (PorcnA-D) as well as mutant transcript in genetrap cell line CSD256 are indicated in black. (B) RT-PCR for *Porcn* in ES cells showing absence of *Porcn* transcript in CSD256 genetrap ES cell line as compared to parental wildtype E14 ES cell line. RT-PCR for *H2afz* was used as housekeeping control. (C) Tcf/Lef-Luciferase assay for canonical Wnt signaling in *Porcn* wt (E14) and mutant (CSD256) ES cells. *Porcn* mutant ES cells show only basal Luciferase activity after transfection with Wnt3a expression plasmid. Increased Luciferase activity was observed in CSD256 by co-transfection of a mixture of PorcnA-D isoforms along with Wnt3a. (D) Individual isoforms of Porcn were tested for their ability to rescue Wnt signaling in a Tcf/Lef-Luciferase assay. All *Porcn* splice variants show similar activity levels of Luciferase. (E) Tcf/Lef-Luciferase assay of Wnt3a point-mutants in *Porcn* wt and mutant ES cells. While wt Wnt3a shows strong upregulation of Luciferase activity in wt cells, Wnt3aC77A shows residual activity. Wnt3a S209 and double mutant show only basal Luciferase activity. In *Porcn* mut cells, all wt and point-mutant Wnt3a constructs failed to elicit an upregulation of Tcf/Lef-Luciferase activity. (F) Paracrine Tcf/Lef-Luciferase assay shows that *Porcn* is only required in Wnt signal secreting cells. Expression of Wnt3a in wt ES cells (Sender) leads to significant up-regulation of Tcf/Lef-Luciferase activity, whereas expression of Wnt3a in mutant cells (Sender) had no effect on canonical Wnt reporter activity as compared to mock (EYFP) transfection, independent of the genotype of Tcf/Lef-Luciferase transfected recipient ES cells. $^{**}p<0.01$, $^{*}p<0.05$, ns = not significant, error bars display standard error, wt = wildtype, mut = mutant.

assays (Veeman et al., 2003) in ES cells (Fig. 2C). Mock-transfected *Porcn* null cells (CSD256) showed reduced luciferase expression compared to the parental wildtype cell line (E14Tg2a.4, Fig. 2C). Upon transfection with a Wnt3a overexpression plasmid, luciferase activity in wildtype cells increased 39-fold, but *Porcn* mutant cells did not show any upregulation and luciferase expression remained at basal levels, indicating defects in canonical Wnt signaling mediated by Wnt3a protein (Fig. 2C). Co-transfection of Wnt3a with a mixture of all four *Porcn* isoforms rescued this defect of mutant cells and caused a 25-fold increase in luciferase activity, but had no significant effect on the Wnt3a-mediated induction in wildtype cells (Fig. 2C). Transfection with the *Porcn* mixture alone had no effect on luciferase activity in wildtype or mutant cells (Fig. 2C).

We next tested whether Wnt3a palmitoylation is catalyzed by a specific *Porcn* isoform; Wnt3a and individual *Porcn* isoforms (A-D) were over-expressed in *Porcn* null ES cells and canonical Wnt activity was measured using the Tcf/Lef-luciferase assay (Fig. 2D). All of the *Porcn* isoforms were able to rescue the *Porcn* null defect in ES cells and no significant differences could be detected between cells transfected with the mixture of isoforms or individual isoforms (Fig. 2D). This does not preclude some isoform-specific effect on other Wnt ligands, but suggests considerable overlap in function of *Porcn* isoforms.

In order to confirm that lack of Wnt3a palmitoylation causes the same effects in our Tcf/Lef-luciferase assays, we over-expressed Wnt3a or point-mutant variants that cannot be lipid-modified (C77A, S209 and double mutant) in wildtype and *Porcn* mutant ES cells (Fig. 2E). As expected, over-expression of wildtype Wnt3a led to a strong increase (70-fold) in luciferase activity (Fig. 2E). Consistent with results obtained in other systems (Doubravska et al., 2011), the S209A and double-mutant Wnt3a showed no upregulation of reporter activity at all, whereas Wnt3aC77A retained some activity and showed a 6-fold increase in Wnt reporter activity compared to mock-transfected cells (Fig. 2E). In *Porcn* mutant ES cells, none the Wnt3a constructs showed any activity over basal levels (Fig. 2E). These results are consistent with a previous report showing that S209 is not acylated upon knock-down of *Porcn* (Takada et al., 2006). Whether *Porcn* is also required for palmitoylation of C77 cannot be addressed in this system, as S209 acylation has been reported to be a pre-requisite for C77 palmitoylation (Doubravska et al., 2011).

We next investigated whether *Porcn* function is required cell autonomously (Fig. 2F). The secreting cell population was transfected with EYFP or Wnt3a expression constructs and mixed with cells transfected with the canonical Wnt reporter construct. Expression of Wnt3a in wildtype cells led to significant upregulation of luciferase activity in both wildtype and mutant recipient cells, as compared to the EYFP control (Fig. 2F). In contrast, *Porcn* mutant cells transfected with Wnt3a were unable to induce a response in either wildtype or mutant recipient cells (Fig. 2F). Thus *Porcn* function is dispensable in Wnt3a signal receiving cells and only required in Wnt3a secreting cells, supporting the role of *Porcn* in Wnt processing and secretion.

Normal Nodal secretion and signaling in Porcn genetrap ES cells

While it has been shown that *Porcn* is required for the acylation of Wnt ligands, potential roles in other signaling pathways have not been investigated extensively. As Nodal signaling is another essential pathway for gastrulation, we investigated whether *Porcn* genetrap ES cells exhibit defects in Nodal secretion and signaling in an autocrine/paracrine assay using the nodal-responsive $(n2)_7$-luciferase construct (Saijoh et al., 2000). Both wildtype and mutant cells showed a moderate increase in luciferase activity upon Nodal overexpression and no statistically significant differences could be detected between the two cell lines (Supplementary Fig. 1). Similarly, no significant differences between the cell lines were found upon stimulation of the pathway with Activin A or inhibition using SB431542 (Supplementary Fig. 1). These results suggest that *Porcn* is not involved in the secretion of Nodal ligand or the reception of Nodal signaling.

Porcn null epiblasts fail to differentiate and establish anterior–posterior identity

In mouse embryogenesis, canonical Wnt signaling is required for gastrulation and the establishment of the anterior–posterior (A–P) axis (Liu et al., 1999). Multiple Wnt ligands are expressed in posterior domains of the embryo and the primitive streak (*Wnt2b*, *Wnt3*, *Wnt3a*, *Wnt5a*, *Wnt8a*, *Wnt11*) and Wnt antagonists such as *Dkk* and *Sfrp2* are expressed by the anterior visceral endoderm (AVE) (Kemp et al., 2005). Consistent with this concept, mutations in the earliest acting Wnt gene, *Wnt3*, causes a lack of primitive streak and the associated ingression of mesodermal cells in the posterior region (Liu et al., 1999).

We have previously shown that *Porcn* null embryos exhibit prolonged expression of the pluripotency marker *Oct4* (Cox et al., 2010), suggesting that *Porcn* null embryos phenocopy *Wnt3* mutant embryos (Liu et al., 1999). In order to determine the phenotype of *Porcn* null embryos in more detail, we analyzed expression patterns of several marker genes at gastrulation stages. As males generated from *Porcn* null ES cells die *in utero* and hence cannot be used to generate a viable mouse line, *Porcn* null embryos were generated by aggregation of mutant ES cells with wildtype GFP-marked host embryos. Only those embryos in which the entire epiblast was ES-derived (40% of dissected aggregation embryos), as judged by absence of GFP expression, were used for this analysis.

As *Wnt3* mutant embryos display a gastrulation phenotype, we analyzed the expression of early gastrulation markers at E6.5 by *in situ* hybridization, to determine whether gastrulation was initiated in *Porcn* null embryos. *Wnt3* expression on the posterior side of the embryo (Supplementary Fig. 2A) precedes primitive streak formation and appears normal in *Porcn* aggregation embryos (n = 3/4, Supplementary Fig. 2B). Further, all embryos analyzed showed expression of *Cer1* (n = 4, Supplementary Fig. 2C and D) and *Lhx1* (n = 5, Supplementary Fig. 2E–F′) in the AVE, confirming the proper formation of this anterior signaling center required for gastrulation. Several embryos also showed posterior embryonic *Lhx1* expression (n = 3/5, Supplementary Fig. 2F′). Although this expression region was smaller than in control embryos (Supplementary Fig. 2E), its presence suggests the initiation of gastrulation.

At later stages, *Wnt3* marks the primitive streak (Fig. 3A) and is expressed in ectopic patches throughout the *Porcn* mutant embryo proper at both E7.5 (n = 4) and E8.5 (n = 5) (Fig. 3B and D). As *Porcn* is required for the lipid modification of Wnt3, this expression is not expected to result in secretion of functional Wnt3 ligand. In order to determine whether the canonical Wnt signaling cascade was active in *Porcn* null embryos, we used *Axin2*, a direct target and negative feedback regulator of canonical Wnt signaling as a read-out (Jho et al., 2002). *Axin2* expression was detected at lower levels and in an ectopic, patchy pattern compared to wildtype control embryos (n = 16), suggesting that canonical Wnt signaling was considerably reduced in *Porcn* mutant embryos (Fig. 3E-H).

Brachyury (T) is also a direct target of canonical Wnt signaling (Yamaguchi et al., 1999) and required for gastrulation in the mouse embryo where it marks the primitive streak at E7.5 (Fig. 3I) and axial mesoderm and the tailbud at E8.5 (Fig. 3K). In the majority of *Porcn* null aggregation embryos (20/24), *T* was only detectable in 1–3 small ectopic patches at E7.5 (n = 16) and E8.5 (n = 8) (Fig. 3J and L). The remaining embryos showed either a normal looking primitive streak (2/24) or complete absence of *T* (2/24). These observations, in combination with the observed patches of mesodermal *Lhx1* expression at E6.5 (Supplementary Fig. 2F′), suggest that primitive streak formation is initiated, but a fully developed streak is not formed. Expression of *HoxB1* was undetectable in all *Porcn* null embryos

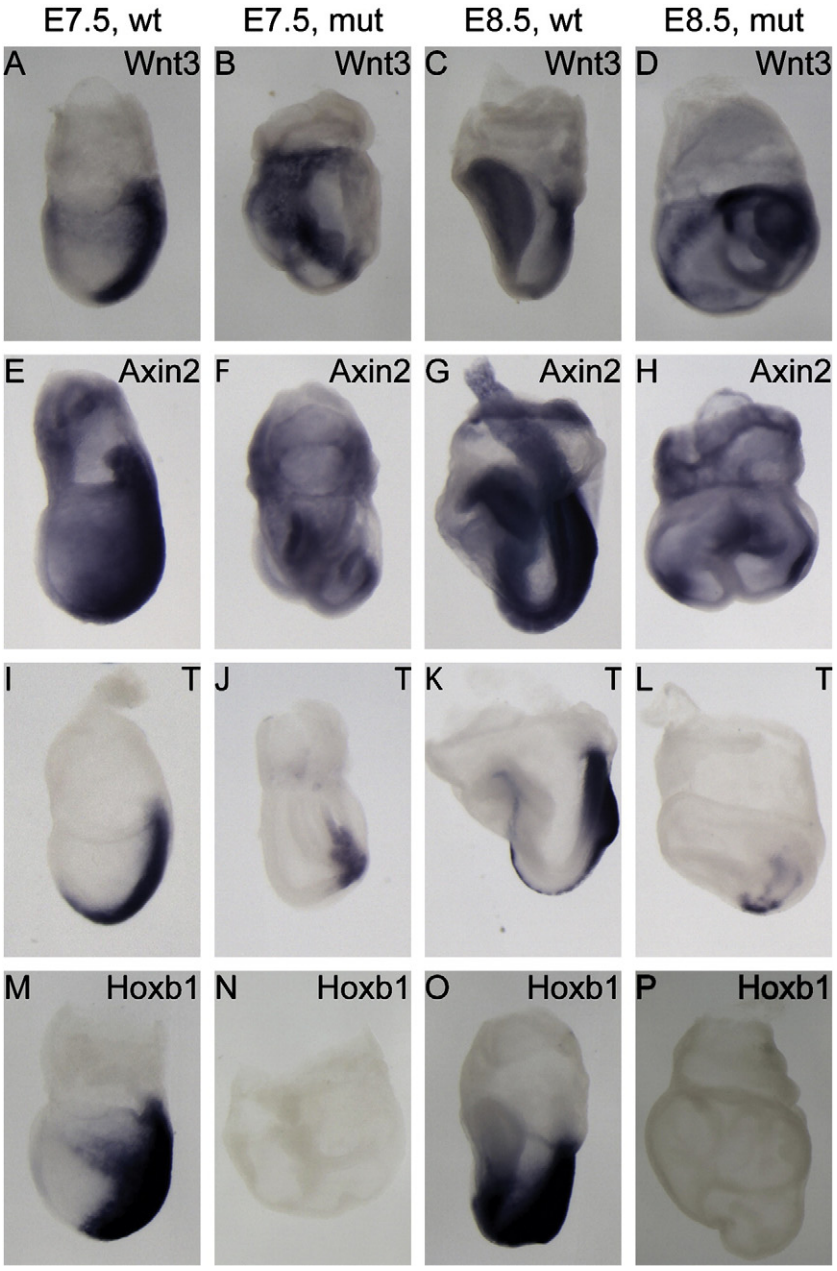

Fig. 3. Analysis of posterior marker gene expresssion in *Porcn* genetrap aggregation embryos. Comparison of expression of posterior patterning genes*Wnt3* (A-D), *Axin2* (E-H), *Brachyury (T)* (I-L) and *Hoxb1* (M-P) in wildtype and *Porcn* genetrap aggregation embryos. Expression of genes was visualized by whole mount RNA *in situ* hybridization. Wildtype embryos are oriented with their anterior to the left. Mutant embryos cannot be oriented, due to a lack of discernible markers or structures. At E7.5, *Wnt3* (A), canonical Wnt signaling target *Axin2* (E) and *T* (I) are expressed in the primitive streak, whereas *HoxB1* (M) marks posterior cell fates in wildtype embryos. *Porcn* mutants display a reduced and ectopic expression of *Wnt3* (B) as well as *Axin2* (F). Expression of *T* (J) is strongly reduced and *HoxB1* (N) is entirely absent. At E8.5, *Wnt3* expression is restricted to the developing neural tissues and the tailbud in wildtype embryos (C), but expressed ectopically in mutant embryos (D). *Axin2* expression at E8.5 is ectopic and strongly reduced in *Porcn* mutant embryos (H) as compared to wildtype embryos (G). *Brachyury (T)* marks the axial mesoderm and tailbud in E8.5 wildtype embryos (K), but is strongly reduced in *Porcn* mutants (L). Posterior cell fate marker *HoxB1* (O) is undetectable in *Porcn* mutant embryos.

analyzed (n = 7, Fig. 3 M-P), confirming the absence of posterior cell fates normally induced by posteriorizing Wnt signals.

As the posterior markers *T* and *Hoxb1* were markedly reduced or absent, we next examined the expression of the anterior markers *Hesx1* and *Otx2* in order to determine if anterior neuro-ectodermal cell fate was established properly or possibly expanded due to a lack of posteriorizing signals. *Porcn* null embryos lacked *Hesx1* expression entirely or showed a strongly reduced expression (n = 9, Fig. 4B). In contrast, strong *Otx2* expression was detectable throughout the embryo proper (n = 9, Fig. 4D and F). In wildtype embryos, *Otx2* is dynamically expressed, marking the epiblast (E5.0), anterior visceral endoderm (AVE, E6.5) or anterior neuroectoderm (E7.5, E8.5, Fig. 4C and E). As previously described (Cox et al., 2010), *Porcn* null embryos maintain a strong expression of *Oct3/4 (Pou5f1)* in the embryonic ectoderm.

While the expression patterns of *Wnt3*, *Axin2* and *T* suggest that the primitive streak may be initiated at E7.5 but not maintained, *Porcn* null embryos fail to establish posterior cell fates based on *HoxB1* expression. The strong expression of *Oct3/4* (Cox et al., 2010) and *Otx2* is likely a continuance of the early epiblast expression from E5.0 rather than an expansion of anterior fates. This conclusion is supported by the observation of the reduction in *Hesx1* expression.

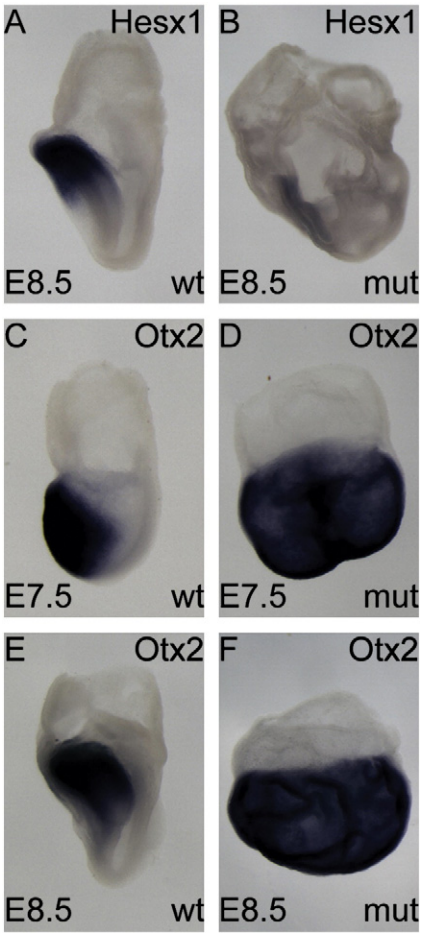

Fig. 4. Analysis of anterior marker gene expresssion in *Porcn* genetrap aggregation embryos. Comparison of expression of anterior patterning genes *Hesx1* (A, B) and *Otx2* (C-F) in wildtype and *Porcn* genetrap aggregation embryos. Expression of genes was visualized by whole mount RNA *in situ* hybridization. Wildtype embryos are oriented with their anterior to the left. Mutant embryos cannot be oriented, due to a lack of discernible markers or structures. *Hesx1* marks the anterior neuroectoderm in wildtype embryos (A) and is strongly reduced (B) or entirely absent in *Porcn* mutant embryos. *Otx2* expression in wildtype embryos is restricted to the AVE at E7.5 (C) and the anterior neuroectoderm at E8.5 (E), but is expressed throughout the entire embryo proper of *Porcn* mutant embryos at both E7.5 and E8.5 (D,F).

Porcn genetrap ES cells fail to differentiate into mesoderm and endoderm derivatives in vitro

In order to assess the differentiation properties of *Porcn* mutant cells in more detail, we studied the differentiation of mutant ES cells as embryoid bodies *in vitro* (Gadue et al., 2005). It has previously been shown that induction of Flk1 (VEGFR2), a marker expressed in the lateral plate mesoderm, is dependent on canonical Wnt signaling (Nostro et al., 2008). To test whether *Porcn* genetrap ES cells can generate Flk1-expressing mesoderm, we differentiated *Porcn* wildtype and null ES cells as embryoid bodies (EBs) *in vitro* in serum-free conditions. Mesoderm formation was induced by addition of Bmp4 (0.5 ng/ml) and Activin A (5 ng/ml) after 48 h and EBs were cultured for an additional 48 h before flow-cytometric analysis (Fig. 5A).

Using SSEA-1 as a marker for ES and epiblast cells and Flk1 as a marker for mesoderm, we could observe that approximately 40% of wildtype cells downregulated SSEA-1 and expressed Flk1 (Fig. 5B), indicating successful mesoderm formation. In contrast, Flk1$^+$ cells were absent entirely in *Porcn* mutant cells (Fig. 5B) and SSEA-1 expression remained high (Fig. 5B and Supplementary Fig. 1).

Absence of Flk1$^+$ cells was also observed in wildtype EBs upon addition of canonical Wnt inhibitor Dkk-1 (300 ng/ml, Fig. 5G and H) or Porcn inhibitor IWP-2 (1 μM, Fig. 5C and D), indicating that the observed defect is in fact dependent on canonical Wnt signaling and the specific function of *Porcn*. In order to circumvent the block in differentiation of *Porcn* mutant cells, we added the Gsk3 inhibitor CHIR99014 (3 μM) to differentiating EBs 6 h after induction with Bmp4 and Activin A. Inhibition of Gsk3 stabilizes free beta-catenin, which is then translocated to the nucleus where it can bind Tcf/Lef1 and activate gene transcription. EBs treated with Gsk3 inhibitor showed downregulation of SSEA-1 and a significant proportion of cells expressing Flk1 were observed in both wildtype (22.60%, Fig. 5E) and mutant cells (32.91%, Fig. 5F). The higher amount of Flk1$^+$ cells in mutant EBs may be due to different kinetics of Flk1 induction between these two cells lines or the failure of *Porcn* mutant cells to secrete a negative modulator of Flk1 induction. Likely candidates would be non-canonical Wnts that may require *Porcn* for their secretion.

In contrast to activation of the canonical Wnt signaling cascade at the level of Gsk3, addition of Wnt3a (up to 100 ng/ml) protein had no effect on mutant EB differentiation (Supplementary Fig. 3). These observations suggest a positive feedback loop, in which a trigger amount of exogenous Wnt3a results in the transcription of multiple other canonical Wnts that are required for gastrulation events. Similar observations have been made for Wnt3 in an embryocarcinoma model (Marikawa et al., 2008).

We further tested whether EBs could be induced to form CXCR4$^+$ endoderm upon induction with Activin A alone at high concentrations (100 ng/ml, Fig. 5I and J) (Morrison et al., 2008; Yasunaga et al., 2005). While wildtype EBs showed downregulation of SSEA-1 and up to 30% of cells became CXCR4$^+$, no CXCR4$^+$ cells were observed in *Porcn* null embryoid bodies (Fig. 5I and J). These results indicate absence of both endoderm and mesoderm in *Porcn* null EBs *in vitro*.

Expanding the analysis of *in vitro* differentiation, we performed quantitative Real-time PCR analysis of ES cells (EB day 0, Fig. 6A), EBs before induction with Bmp4 and Activin A (EB day 2, Fig. 6B) and at the time of flow-cytometric analysis (EB day 4, Fig. 6C). Using a panel of marker genes for pluripotency (*Fbx15, Klf4, Oct4*), epiblast (*E-cadherin, Fgf5*), neuroectoderm (*Gbx2, Sox1, Pax6, Nestin*), mesoderm (*T, Tbx6, Pdgfra, Mixl*), endoderm (*Sox17, Cer1, Cxcr4*) as well as Wnt pathway members (*Wnt5a, Axin2, c-Myc*), we assessed the phenotype of *Porcn* mutant ES cells and EBs on a molecular level (Fig. 6A-C).

At EB day 0 and EB day 2 (Fig. 6A and B), only minor changes in gene expression levels were detected that were not statistically significant for the majority of genes assessed (Student's *t*-test, $p>0.05$), suggesting that *Porcn* mutant ES cells had normal expression levels compared to wildtype ES cells and successfully adopted an epiblast-like state at EB day 2. Upon induction with Bmp4 and Activin A (EB day 4, Fig. 6C), the difference between *Porcn* wildtype and mutant cells in pluripotency, epiblast and neuroectoderm marker gene expression remained statistically not significant with the exception of the downregulation of *Klf4* and *Gbx2*. In contrast, all mesodermal and endodermal marker genes were significantly downregulated with *Mixl* being the most extreme. Further, the primitive streak marker *Wnt5a*, as well as the direct canonical Wnt signaling targets *Axin2* and *c-Myc* were also significantly downregulated. Visual assessment of differentiating EBs as well as the proportion of dead cells detected during flow-cytometric analysis by propidium iodide staining (data not shown) suggests no major defects in cell proliferation or apoptosis under these conditions.

Taken together, our *in vitro* data suggest that *Porcn* mutant ES cells fail to differentiate into endodermal and mesodermal derivatives, consistent with the requirement for canonical Wnt signaling in the induction of these germ layers.

Discussion

We have recently discovered *Porcn* in a screen for X chromosome linked embryonic lethal mutations in the mouse (Cox et al., 2010).

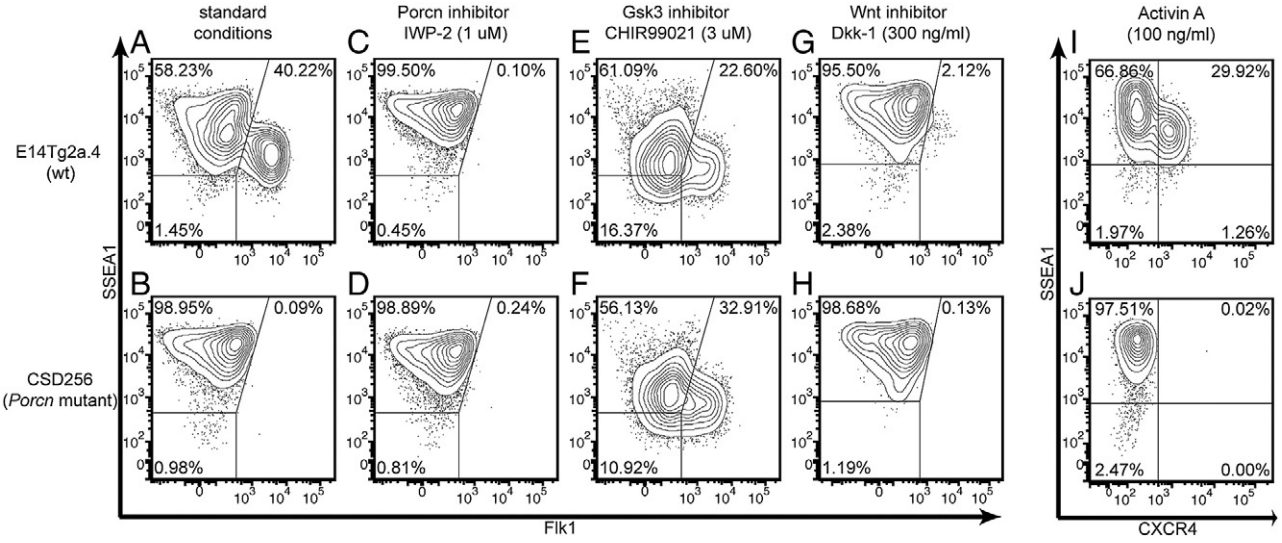

Fig. 5. *Porcn* mutant embryoid bodies (EBs) fail to generate Flk1$^+$ mesoderm and CXCR4$^+$ endoderm *in vitro*. *Porcn* wildtype (E14Tg2a.4) and mutant (CSD256) ES cells were differentiated *in vitro* as embryoid bodies in serum-free media with 0.5 ng/ml Bmp4 and 5 ng/ml Activin A to generate mesoderm cells. After 4 days of differentiation, EBs were dissociated and analyzed for the expression of cell surface markers SSEA-1 (ES/epiblast cells), Flk1 (VEGFR2, lateral plate mesoderm) and CXCR4 (endoderm) by flow-cytometry. While wildtype ES cells generated Flk1$^+$ SSEA1^{low} mesoderm (A, 40.22%), this population was entirely absent in *Porcn* mutant cells and SSEA1 expression remained high (B, 0.09%). No Flk1$^+$ SSEA1^{low} cell population was observed in wildtype (C, 0.10%) or mutant (D, 0.24%) cells differentiated in the presence of the Porcn chemical inhibitor IWP-2. Addition of the Gsk3 inhibitor CHIR99021 allowed differentiation into Flk1$^+$ SSEA1^{low} mesoderm of both wildtype (E, 22.60%) and mutant (F, 32.91%) cells. No Flk1$^+$ SSEA1^{low} cell population was observed in wild type cells (G, 2.12%) or mutant (H, 0.13%) cells differentiated in the presence of the canonical Wnt signaling inhibitor Dkk-1. Differentiation of cells without Bmp4 and with high levels of Activin A (100 ng/ml) directs differentiation of wildtype ES cells towards CXCR4$^+$ endoderm (I, 29.92%). *Porcn* mutant cells fail to generate CXCR4$^+$ endoderm (J, 0.02%) under these conditions.

Here, we have expanded the analysis of *Porcn* mutant embryos derived from aggregations of *Porcn* null ES cells with wildtype donor embryos. While it has previously been established in lower organisms and *in vitro* systems that *Porcn* is required for the lipid-modification of Wnt ligands, we present the first analysis showing that mammalian embryos and ES cells require *Porcn* for the secretion of functional ligands to induce canonical Wnt signaling responses. The defects caused by *Porcn* ablation in mouse embryos also provide insight into the lethality of human *PORCN* mutant male embryos.

It has been established *in vitro* using siRNA and chemical inhibition that *Porcn* is required for acylation and secretion of Wnt3a (Takada et al., 2006) and Wnt5a ligands (Chen et al., 2009). Tcf/Lef-luciferase assays show that *Porcn* is further required for activity of Wnt1 and Wnt2 in mouse L-cells (Chen et al., 2009), suggesting that *Porcn* function is required for all Wnt ligands. In keeping with these observations, we have been able to show that genetic ablation of *Porcn* leads to a failure to generate functional secreted Wnts as assessed in Tcf/Lef-luciferase assays in mouse ES cells. By using appropriate mixtures of wildtype and mutant cells, we were able to definitively demonstrate an exclusive requirement for *Porcn* in Wnt producing cells, whereas *Porcn* is dispensable in Wnt receiving cells.

Consistent with previously established roles for canonical Wnt signaling in establishing endodermal and mesodermal cell fates, *Porcn* mutant ES cells failed to generate these two germ layers after differentiation in embryoid bodies *in vitro*. Treatment of wild type cells with a chemical inhibitor of *Porcn* (IWP-2) or an extracellular Wnt inhibitor (Dkk) phenocopied the *Porcn* mutant differentiation defect. However the defect could be rescued by treatment of mutant cells with a Gsk3 inhibitor (CHIR99014), which would stabilize beta-catenin and activate the canonical Wnt signaling pathway in mutant cells. These results are all consistent with a specific role for *Porcn* in promoting the correct processing and secretion of Wnts.

Interestingly, however, addition of exogenous Wnt3a protein to mutant EB cultures did not rescue the mutant defects. Since our cell mixing studies had conclusively excluded the possibility that *Porcn* is somehow required in Wnt receiving cells, this result suggests that exogenous Wnt alone is not sufficient to activate the full downstream mesoderm differentiation pathway. It has previously been shown that Wnt3 is regulated in a positive feedback loop in differentiating P19 embryonal carcinoma cells and is further required to induce other Wnts typically expressed in the primitive streak (Marikawa et al., 2008). We suggest that addition of Wnt3a to EB cultures triggers a positive feedback loop, which results in the expression of multiple Wnt ligands required for gastrulation. In *Porcn* mutants, this feedback loop will be non-functional since *Porcn* is required for the secretion or function of those ligands as well. Addition of Wnt3a will thus not result in the secretion of Wnt ligands from mutant cells and fail to induce gastrulation. In contrast, when canonical Wnt signaling is activated by Gsk3 inhibition, this positive feedback loop is not required for the generation of Flk1$^+$ cells. Further, *Porcn* mutant cells would also fail to secrete non-canonical Wnts, which could be required as negative regulators of Flk1 expression, explaining the increase in Flk1$^+$ cells in EBs after Gsk3 inhibition.

These *in vitro* studies on *Porcn* mutant ES cells strongly suggest that *Porcn* will be required for the earliest Wnt dependent developmental events *in vivo*. This was confirmed by examining the phenotype of *Porcn* mutant ES-derived embryos. Mutant embryos showed major gastrulation defects. *Porcn* mutant epiblast cells fail to specify anterior and posterior tissue fates, but instead remain in an "epiblast-like" state with high levels of *Oct4* (Cox et al., 2010) and *Otx2* transcription. The epiblast continues to proliferate, leading to folds of embryonic ectoderm within the visceral endoderm (Cox et al., 2010). These observations support the role for *Porcn* in Wnt signaling, as *Wnt3*$^{-/-}$ embryos show a similar phenotype (Barrow et al., 2007; Liu et al., 1999). Further, *Gpr177/Wls*, encoding a protein involved in trafficking Wnt ligands from the Golgi to the cell surface, has been knocked out in the mouse and also shows a highly similar phenotype (Fu et al., 2009). Based on the similarity of these three mutants, we can conclude that *Porcn* and *Gpr177* are involved in the generation and secretion of functional Wnt3 ligand *in vivo*.

Canonical Wnt signaling has been implicated in the maintenance of pluripotency of ES cells, yet our data, as well as the absence of pre-implantation phenotypes of beta-catenin and Wnt ligand knock-out mice (van Amerongen and Berns, 2006), indicates that canonical Wnt

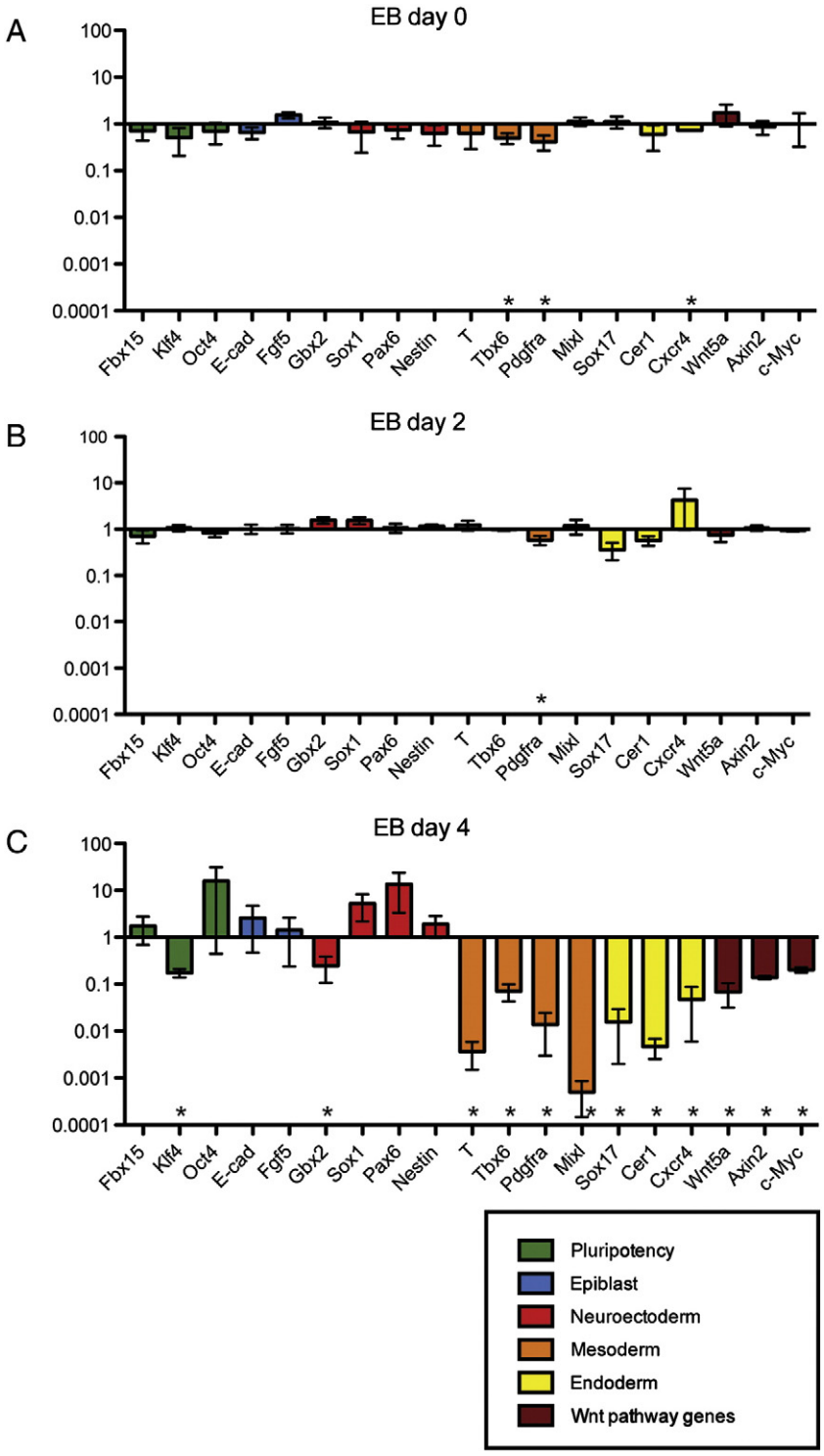

Fig. 6. Gene expression analysis of *Porcn* mutant embryoid bodies (EBs) confirms failure in generation of endodermal and mesodermal derivatives *in vitro*. Quantitative Real-time PCR analysis of in *Porcn* wildtype (E14Tg2a.4) and mutant (CSD256) ES cells differentiated *in vitro* as embryoid bodies in serum-free media. RNA was harvested from ES cells at the time of EB set-up (EB day 0, A), before addition of growth factors Bmp4 and Activin A (EB day 2, B) and at the time of flow-cytometric analysis (EB day 4, C). Bars indicate deviation of gene expression level in *Porcn* mutant cells compared to wildtype cells at the respective time point. Error bars indicate standard errors. Statistical significance indicated by * (Student's *t*-test, $p<0.05$). The selected marker genes show only minor differences in expression levels at EB day 0 (A) and EB day 2 (B), suggesting that *Porcn* mutant ES cells successfully transition into an epiblast-like state (B). At EB day 4 (C), changes in pluripotency, epiblast and neuroectoderm marker gene expression are statistically not significant, with the exception of *Klf4* and *Gbx2*. In contrast, all markers for mesodermal and endodermal derivatives were significantly reduced as compared to wildtype EBs, indicating absence or strong reduction in those cell types. Canonical Wnt signaling targets *Axin2* and *c-Myc* were also significantly reduced.

signaling is not required for the maintenance of pluripotency in the inner cell mass of the blastocyst. We suggest that the *in vitro* effects of Wnt activation are based on its ability to inhibit neural differentiation of ES cells (Kielman et al., 2002; Watanabe et al., 2005). Thus while Wnts may not be required for pluripotency *in vivo*, Wnt signaling *in vitro* can be used to maintain ES cells in a pluripotent state by blocking neural differentiation, if other factors in the media, such as LIF (Ogawa et al., 2006), counteract its mesoderm-inducing capabilities.

On a molecular level, our *in vitro* studies also support a role for *Porcn* in the acylation of S209 of Wnt3a. Whether only a subset or all mammalian Wnt ligands are lipid-modified by *Porcn* is currently unknown. All mammalian Wnt proteins tested to date interact with *Porcn* in over-expression studies (Tanaka et al., 2000) and the two palmitoylated residues are conserved in all mouse Wnt ligands (Takada et al., 2006), but a systematic analysis of *Porcn*-dependence of Wnt ligands is lacking.

In the ES-derived *Porcn* mutant embryos, expression of canonical Wnt targets, such as *Axin2* and *T*, was reduced but not absent, suggesting that there might not be a complete block in Wnt function in these embryos. In contrast to zygotic mutant embryos, the extraembryonic tissues derived from trophectoderm and primitive endoderm in the analyzed aggregation embryos are derived from the wild type donor embryos. Hence, there could be some residual Wnt secretion from these tissues. *Porcn* was not expressed strongly in the extraembryonic tissues of the peri-gastrulation mouse embryo but it was expressed in some regions of the visceral endoderm, such as the AVE at E6.5. The AVE, however, has not been described as a source for Wnt ligands so far, but has been shown to secrete Wnt inhibitors Dkk and sFRP. Transient expression of *Wnt3* in the region proximal to the AVE has been reported (Rivera-Pérez and Magnuson, 2005). While it is possible that *Porcn* has no functional role in the AVE, it opens the possibility that *Porcn* may have Wnt independent functions or substrates.

Due to its significant role in gastrulation, we investigated whether *Porcn* could be involved in Nodal signaling, but were unable to detect defects in Nodal secretion or downstream signaling in the ES cell system. Using a chemical *Porcn* inhibitor, another study has excluded effects of *Porcn* on Notch and Sonic Hedgehog (Shh) signaling (Chen et al., 2009). Together, these data support that *Porcn* is exclusively required for acylation of Wnt ligands.

To allow for detailed, time- or tissue-specific analyses of *Porcn's* role throughout mouse embryonic development and adult tissue homeostasis, a conditional *Porcn* allele avoiding embryonic lethality of founder males will be required. These analyses may also shed light on *Porcn*'s substrates, and whether these include all Wnts and even non-Wnt ligands.

PORCN mutations in humans

While *PORCN* heterozygous human females present with FDH, zygotic mutant males have not been observed to date, suggesting a requirement for *PORCN* in human embryonic development. The time-point or cause of the male embryonic lethality is unknown. Using *Porcn* mutant mouse ES cells to generate aggregation embryos, we were able to establish a model for *Porcn* mutant male mammals. Based on this model, we propose that embryonic lethality of *PORCN* mutant male human embryos is due to a gastrulation defect and a failure to form endodermal and mesodermal derivatives.

Supplementary materials related to this article can be found online at doi:10.1016/j.ydbio.2011.04.029.

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