Development 140, 2961-2971 (2013) doi:10.1242/dev.094458

Porcn-dependent Wnt signaling is not required prior to mouse gastrulation

Steffen Biechele[1,2], Katie Cockburn[1,2], Fredrik Lanner[1,*], Brian J. Cox[1,‡] and Janet Rossant[1,2,§]

SUMMARY

In mice and humans the X-chromosomal porcupine homolog (*Porcn*) gene is required for the acylation and secretion of all 19 Wnt ligands and thus represents a bottleneck for all Wnt signaling. We have generated a mouse line carrying a floxed allele for *Porcn* and used zygotic, oocyte-specific and visceral endoderm-specific deletions to investigate embryonic and extra-embryonic requirements for Wnt ligand secretion. We show that there is no requirement for Porcn-dependent secretion of Wnt ligands during preimplantation development of the mouse embryo. Porcn-dependent Wnts are first required for the initiation of gastrulation, where Porcn function is required in the epiblast but not the visceral endoderm. Heterozygous female embryos, which are mutant in both trophoblast and visceral endoderm due to imprinted X chromosome inactivation, complete gastrulation but display chorio-allantoic fusion defects similar to *Wnt7b* mutants. Our studies highlight the importance of Wnt3 and Wnt7b for embryonic and placental development but suggest that endogenous Porcn-dependent Wnt secretion does not play an essential role in either implantation or blastocyst lineage specification.

KEY WORDS: *Porcn*, Wnt, Gastrulation, Blastocyst, MBOAT

INTRODUCTION

During development, communication between cells is essential to generate a functional organism with all tissue types in appropriate spatial and temporal association. One of the conserved signaling cascades employed for cell communication is the Wnt signaling pathway, which is required for embryonic development as well as tissue homeostasis (Logan and Nusse, 2004). Both inactivation and ectopic activation of this pathway have detrimental effects ranging from embryonic lethality to cancer in mammals (Clevers and Nusse, 2012; Logan and Nusse, 2004). The active signaling molecules are the Wnt ligands: a family of acylated proteins that can activate intracellular signaling cascades in signal-receiving cells (Logan and Nusse, 2004).

In mammals, 19 Wnt ligands are encoded in the genome. Binding of Wnt ligands to the Frizzled (Fzd) receptors generates several parallel but interconnected downstream intracellular signaling cascades (Niehrs, 2012). Activation of the canonical Wnt signaling pathway protects β-catenin from degradation (Behrens et al., 1998; Hamada et al., 1999; Itoh et al., 1998), leading to accumulation of β-catenin in the nucleus, where it interacts with Tcf/Lef transcription factors to activate the transcription of target genes (Daniels and Weis, 2005).

The secretion of all mammalian Wnt ligands is highly conserved and controlled by post-translational modifications (Herr et al., 2012; Najdi et al., 2012). Palmitoylation of a conserved serine (S209 in Wnt3a) by Porcn (Kadowaki et al., 1996; Takada et al., 2006), a member of the membrane-bound O-acyl transferase (MBOAT) family (Hofmann, 2000), is required for binding of Wnt ligands to Wls (Coombs et al., 2010; Herr and Basler, 2012), a cargo receptor required for transport from the Golgi to the cell surface, as well as binding to Fzd receptors on signal-receiving cells (Janda et al., 2012; Komekado et al., 2007). These functions place *Porcn* in a key position in the Wnt signaling network, as Wnt ligands fail to be secreted efficiently from cells in culture or to activate Fzd-dependent Wnt signaling in the absence of *Porcn* (Galli and Burrus, 2011; Janda et al., 2012; Najdi et al., 2012; Takada et al., 2006), and result in tight control over Wnt signaling by Porcn function in the secreting cells (Biechele et al., 2011; Proffitt and Virshup, 2012). In addition to these roles, Wnt-independent functions for Porcn have recently been observed but remain elusive (Covey et al., 2012).

Genetic ablation of *Porcn* in the mouse is an important tool to address Wnt ligand secretion and redundancy *in vivo*. Redundancy of *Wnt1* and *Wnt3a* has been reported (Ikeya and Takada, 1998), but systematic investigation using classic genetic approaches is laborious due to the embryonic lethality of numerous Wnt ligand and pathway component mutants (van Amerongen and Berns, 2006). We and others have previously shown that embryos lacking *Porcn* specifically in the epiblast fail to gastrulate (Barrott et al., 2011; Biechele et al., 2011). Although these data establish the role in the embryo proper, they fail to address extra-embryonic and preimplantation functions of *Porcn*. Zygotic deletion of *Porcn* has been reported but not investigated in detail (Liu et al., 2012). *Porcn* functions in the preimplantation embryo are of particular interest, as canonical Wnt signaling and β-catenin have been implicated in the maintenance of pluripotent mouse embryonic stem cells (ESCs) (ten Berge et al., 2011; del Valle et al., 2013; Faunes et al., 2013; Habib et al., 2013), which are derived from the inner cell mass (ICM) of the mouse blastocyst.

We have generated a mouse line carrying a floxed allele for *Porcn* as a tool to ablate Wnt ligand secretion, and used zygotic, oocyte-specific and visceral endoderm (VE)-specific deletions to investigate embryonic and extra-embryonic requirements for Porcn-dependent Wnt signaling in early mouse development. We

[1]Program in Developmental and Stem Cell Biology, Hospital for Sick Children Research Institute, Toronto, ON, M5G 1X8, Canada. [2]Department of Molecular Genetics, University of Toronto, Toronto, ON, M5S 1A8, Canada.

*Present address: Department of Clinical Science, Intervention and Technology, Karolinska Institutet, Stockholm, SE-171 77, Sweden
‡Present address: Department of Physiology, University of Toronto, ON, M5S 1A8, Canada
§Author for correspondence (janet.rossant@sickkids.ca)

Accepted 9 May 2013

demonstrate that *Porcn*-dependent embryonic Wnt signals are not required in preimplantation development, nor for implantation itself. Consistent with numerous canonical Wnt pathway mutants, our study identifies gastrulation as the first Porcn/Wnt-dependent event in embryonic development. Taking advantage of extra-embryonic imprinted X chromosome inactivation in heterozygous females, we show that extra-embryonic *Porcn* function is required for chorio-allantoic fusion.

MATERIALS AND METHODS

Generation of a *Porcn* floxed mouse line

The *Porcn* locus was targeted in G4 ESCs (George et al., 2007), introducing loxP sites flanking exon 3 and an FRT-flanked neomycin resistance cassette (Fig. 1B). Homologous recombination was confirmed by Southern blot (supplementary material Fig. S1A,B) and long-range PCR (supplementary material Fig. S1C) with complete sequencing of amplicons. After excision of the resistance cassette, founder animals were generated from clone H4D4. Animals were genotyped by PCR using the following primers (5′-3′): PorcnRecF1, CTGTTAAACCAAGACATGACCTTCA; PorcnRecR1, TAACTAGGACGCTTTGGGATAGGAT; and PorcnRecR3, GTTCTGCC-TTCCTAACCCATATAAC.

Mouse alleles and genetic backgrounds

All animal experiments were performed in a specific pathogen-free environment at the Toronto Centre for Phenogenomics (TCP) and all procedures were approved by the Institutional Animal Care Committee in accordance with guidelines by the Canadian Council for Animal Care (CCAC). Unless indicated otherwise, experiments were performed using outbred ICR mice carrying the *Porcn* floxed allele and/or the following transgenes; *D4/XEGFP* [*Tg(GFPX)4Nagy*] (Hadjantonakis et al., 1998), *pCX-NLS-Cre* [*Tg(ACTB-cre)1Nagy*] (Belteki et al., 2005), *Tcf/Lef-lacZ* (Mohamed et al., 2004), *Hhex-EGFP* [*Tg(Hhex-EGFP)#Rbe*] (Rodriguez et al., 2001), *Zp3-Cre* [*Tg(Zp3-Cre)3Mrt*] (Lewandoski et al., 1997), *Ttr::Cre* [*Tg(Ttr-cre)1Hadj*] (Kwon and Hadjantonakis, 2009) and *Ctnnb1*$^{lox(ex3)}$ (*Ctnnb1*^{tm1Mmt}) (Harada et al., 1999). For blastocyst studies, the incipient congenic (>F5) *Porcn* floxed (C57BL/6J) allele was deleted using a different *Zp3-Cre* allele [*Tg(Zp3-cre)93Knw/J*] (de Vries et al., 2000). Genotyping was performed using the REDExtract-N-Amp Tissue PCR Kit (Sigma) using the primers indicated in original publications. All genotypes mentioned indicate the maternal allele first (i.e. *Gene*$^{mat/pat}$).

Postimplantation embryo collection, staining and imaging

Embryos were obtained from natural timed matings and were dissected in PBS. Whole-mount *in situ* hybridization and β-galactosidase staining were performed as described (Biechele et al., 2011; Cox et al., 2010). Integrin alpha 4 immunohistochemistry was performed as described (Daane et al., 2011).

Sex-separated and diapause pregnancies

Embryos were generated by natural mating of *Porcn*$^{lox/lox}$*; Zp3-Cre(Mrt)*$^{+/tg}$ females to *XEGFP*$^{tg/Y}$ males. Embryos were harvested at E3.5 [or EDG10 after diapause induction (Hunter, 1999)], sexed based on EGFP fluorescence (Hadjantonakis et al., 1998), transferred separately into E2.5 pseudo-pregnant females and recovered from uteri 5 days later (E7.5).

Preimplantation embryo imaging

Embryos were generated by natural mating, recovered in M2 (Specialty Media, Chemicon) at E3.5, and cultured in KSOM (Specialty Media, Chemicon) under mineral oil (Sigma) at 37°C and 5% CO_2 in air for 24 hours (E4.5). Fixation and immunostaining were performed as described (Stephenson et al., 2010) using the antibodies specified in supplementary material Table S1. Images were captured using a Zeiss Axiovert 200 inverted microscope equipped with a Hamamatsu C9100-13 EM-CCD camera, a Quorum spinning disk confocal scan head and Volocity acquisition software (Perkin Elmer). *z*-stacks were taken at 2 μm intervals with a 20× water-immersion objective (NA=0.75). Images were exported to ImageJ (NIH) for analysis, and cells were manually scored as epiblast, primitive endoderm or trophectoderm based on the position of Hoechst-stained nuclei in the embryo and expression of Nanog, Gata6 and Cdx2. Per genotype, cells of five embryos were quantified and statistically analyzed by chi-square analysis.

Single-cell gene expression analysis

For gene expression analysis, cultured E4.5 embryos were dissociated into single cells as described (Rugg-Gunn et al., 2012). For maternal/zygotic mutants, embryos were dissociated individually and sex (female heterozygous, male mutant) was determined based on expression of Xist, Uty and Ddx3y (supplementary material Table S4). Control embryos (C57BL/6NCrl and *pCX-Nls-Cre*$^{tg/+}$*; Ctnnb1*$^{+/del\ ex3}$) were pooled. Gene expression analysis was performed using 48.48 Dynamic Arrays on the BioMark system (Fluidigm) as described (Rugg-Gunn et al., 2012). Data were analyzed in R, using a modified version of the LogEx method as published by Fluidigm. Samples were normalized by determining the lowest observed detection (LOD) for each probe and subtracting the observed cycle threshold (Ct) values. Median and standard deviations for each probe were calculated. Cells with Ct<5 for control probes (*Actb*, *Gapdh*) were removed. Cells passing this quality control (*mzPorcn*$^{del/Y}$, *n*=35; *Porcn*$^{del/+}$, *n*=49; *Porcn*$^{+/+}$, *n*=93; *Ctnnb1*$^{+/del\ ex3}$, *n*=180; obtained from 8-20 embryos/genotype) were then classified into three lineages using known lineage markers (supplementary material Table S2) by expectation maximization clustering forced to a fit of three populations. Next, the median Ct value for each cell lineage and genotype was calculated. These values were then clustered using the default parameters of the heatmap.2 method from the R library gplots (http://cran.r-project.org/web/packages/gplots/).

ESC experiments

RT-PCR was performed as described (Biechele et al., 2011) with the following primers (5′-3′): PorcnEx2-4F, GGCTGCTTCTTACCATCTGC; PorcnEx2-4R, TGTCCACCATGTGCATCTCAC; PorcnEx4-9F, GTGA-GATGCACATGGTGGAC; and PorcnEx4-9R, ACTGTCAGGTCC-CATTCCAG. Autocrine Tcf/Lef-luciferase assays were performed as described (Biechele et al., 2011).

For flow cytometric analysis, a single-cell suspension of *Porcn*$^{lox/Y}$ (H4D4) or *Porcn*$^{del/Y}$ (H4D4F9) ESCs was plated onto gelatin/serum-coated plates at 10,000 cells/cm^2 in serum-free N2B27 medium supplemented with LIF and 1 μM PD0325901 (Selleck Chemicals) (Nichols and Ying, 2006). In addition, 3 μM CHIR99021 (Selleck Chemicals), DMSO (Sigma) or 1 μM IWP2 (Sigma) was added. Media were changed daily and cells were passaged every 3 days using trypsin (Gibco) and plated at 10,000 cells/cm^2. At every passage, a portion of the cells was analyzed by flow cytometry (antibodies are listed in supplementary material Table S3) using an LSRII flow cytometer (Becton Dickinson) and FACS plots were generated using FlowJo software (Tree Star). The FACS plots shown in supplementary material Fig. S6 were obtained after the third passage.

RESULTS

Generation of a *Porcn* floxed allele

We have previously shown that mice generated by aggregation of *Porcn* mutant ESCs with tetraploid embryos die at gastrulation stages, making it impossible to generate a viable mouse line (Biechele et al., 2011; Cox et al., 2010). In order to circumvent this problem and analyze *Porcn* function during embryonic development in more detail, we generated a conditional *Porcn* allele carrying loxP sites flanking exon 3 (Fig. 1B,C). *Porcn* floxed (*Porcn*$^{lox/Y}$; Fig. 1C) ESCs were used to generate chimeric founder males. Germline transmission of the allele was observed by coat color and PCR genotyping, and heterozygous floxed offspring were used to establish a breeding colony. Both hemizygous and homozygous allele carriers showed no defects in embryogenesis or adult life and exhibit normal fertility on outbred ICR and inbred C57BL/6J backgrounds (F10).

On a molecular level, deletion of exon 3 is predicted to cause a frameshift and premature stop codons leading to nonsense-mediated decay of the mutant transcript. To confirm this, we generated *Porcn*-

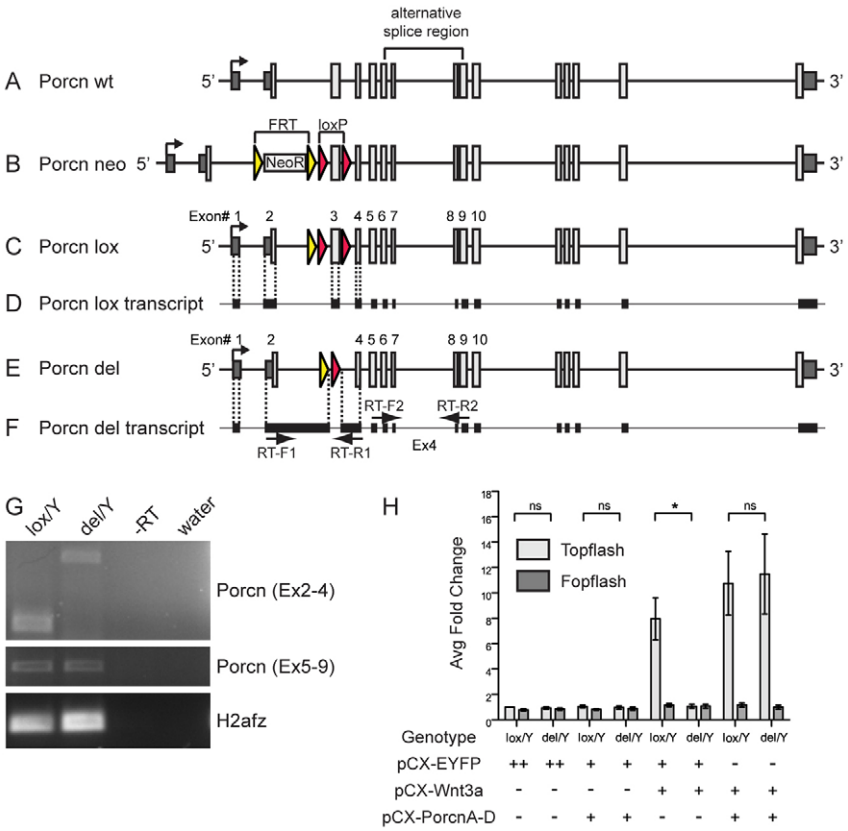

Fig. 1. Generation and characterization of a conditional *Porcn* allele. (**A-F**) Schematic of the mouse *Porcn* locus and genetic manipulations to generate the mutant allele. (A) Wild-type *Porcn* locus. (B) *Porcn neo* allele generated to introduce loxP sites flanking exon 3. (C) *Porcn lox* allele used to generate the mouse line. (D) Transcript generated from the floxed allele. (E) *Porcn del* allele resulting from Cre excision. (F) *Porcn del* transcript exhibits aberrant splicing and inclusion of intron 2/3. (**G**) RT-PCR for *Porcn* from $Porcn^{lox/Y}$ and $Porcn^{del/Y}$ cells. Mutant cells exhibit aberrant splicing including intronic sequences between exon 2 and 4 in place of the excised exon 3 (Ex2-4). *H2afz* was used as a control. Primer locations are indicated in F. –RT, no reverse transcriptase; water, no template. (**H**) Tcf/Lef-luciferase assay. $Porcn^{del/Y}$ ESCs fail to express Tcf/Lef-luciferase in response to Wnt3a transfection, compared with control $Porcn^{lox/Y}$ ESCs. Cotransfection of Wnt3a and Porcn isoforms A-D resulted in similar upregulation of Tcf/Lef-luciferase activity in both $Porcn^{lox/Y}$ and $Porcn^{del/Y}$ ESCs. Control transfections (EYFP or Porcn only) had no effect. The assay was performed with three biological replicates; error bars indicate s.e.m. *P<0.05 (Student's t-test); ns, not significant.

deleted ESCs ($Porcn^{del/Y}$, Fig. 1E) by transient expression of Cre recombinase. Unexpectedly, *Porcn* transcript was still detectable by RT-PCR in *Porcn* mutant ESCs (Fig. 1G), and sequencing of RT-PCR products revealed that deletion of exon 3 caused aberrant splicing between exons 2 and 4, resulting in the inclusion of the majority of the fusion intron in the transcript (Fig. 1F). This mutant transcript contains ten stop codons before exon 4 and should thus not lead to a functional protein product. In order to confirm that $Porcn^{del/Y}$ ESCs are functionally mutant, we performed autocrine Wnt secretion assays based on the canonical Wnt reporter Tcf/Lef-luciferase (Biechele et al., 2011; Veeman et al., 2003). Upon overexpression of Wnt3a, luciferase activity increased 8-fold in $Porcn^{lox/Y}$ ESCs (Fig. 1H). By contrast, $Porcn^{del/Y}$ ESCs showed no upregulation of luciferase activity. This defect could be rescued by cotransfection of *Wnt3a* with a mixture of all four *Porcn* isoforms (Fig. 1H). Control transfections with *EYFP* or *Porcn* in the absence of *Wnt3a* expression plasmid had no effect on luciferase activity. These results show that deletion of *Porcn* exon 3 results in a functionally mutant allele that phenocopies the *Porcn* gene-trap allele (CSD256, BayGenomics) and an independent *Porcn* floxed allele (Barrott et al., 2011) *in vitro*.

Zygotic *Porcn* deletion causes gastrulation defects in hemizygous male embryos

We and others have previously shown that embryos lacking *Porcn* specifically in the epiblast fail to gastrulate (Barrott et al., 2011; Biechele et al., 2011). As these embryos have wild-type extra-embryonic tissues that can act as sources of Wnt signaling (Barrow et al., 2007; Tortelote et al., 2013), we investigated whether zygotic and epiblast-specific mutants differ in phenotype. We generated zygotic *Porcn* mutant embryos by deletion with a ubiquitously expressed Cre recombinase (Belteki et al., 2005). Hemizygous *Porcn* mutant embryos ($Porcn^{del/Y}$) could be recovered up to E7.5 and were detected at the expected Mendelian frequencies, but were smaller than wild-type littermates and lacked amnion and chorion (Fig. 2; supplementary material Fig. S2A,B). There was no excessive folding of the epiblast as seen in embryos generated by aggregation of gene-trap mutant ESCs (Biechele et al., 2011), but the morphology was very similar to that of the published epiblast-specific *Porcn* mutants (Barrott et al., 2011).

Consistent with complete ablation of Wnt secretion, *Porcn* mutant embryos lack a canonical Wnt signaling response based on a *Tcf/Lef-lacZ* reporter allele (Mohamed et al., 2004) at E6.5 (n=2, Fig. 2A′) and E7.5 (n=4, Fig. 2B′), whereas β-galactosidase activity was readily detectable in the primitive streak at both E6.5 (n=5, Fig. 2A) and E7.5 (n=5, Fig. 2B) in wild-type embryos. To extend these observations, we assessed the expression of endogenous targets of canonical Wnt signaling in the primitive streak. Brachyury (*T*) (Arnold et al., 2000; Yamaguchi et al., 1999) and *Wls* (*Gpr177*) (Fu et al., 2009) were both undetectable in mutant embryos (n=6, Fig. 2C′; n=12, supplementary material Fig. S2C′). Consistent with previous suggestions of gastrulation defects (Barrott et al., 2011; Biechele et al., 2011), we were unable to detect the migrating mesoderm marker *Lhx1* (Shawlot et al., 1999) (n=6, supplementary material Fig. S2D′) and the posterior cell fate marker *Hoxb1* (n=4, Fig. 2D′) in *Porcn* mutant embryos. In contrast to the absence of primitive streak and posterior marker genes, $Porcn^{del/Y}$ embryos continued to express the pluripotency marker *Oct3/4* (*Pou5f1* – Mouse Genome Informatics) (n=10, Fig. 2H′) and failed to restrict *Otx2* to the anterior region (n=12, Fig. 2G′), suggesting that these embryos remain in an 'early epiblast-like' state, similar to *Wnt3* mutant embryos (Liu et al., 1999).

It has been shown that the primitive streak is induced by Wnt3 (Barrow et al., 2007; Liu et al., 1999; Tortelote et al., 2013). *Wnt3*

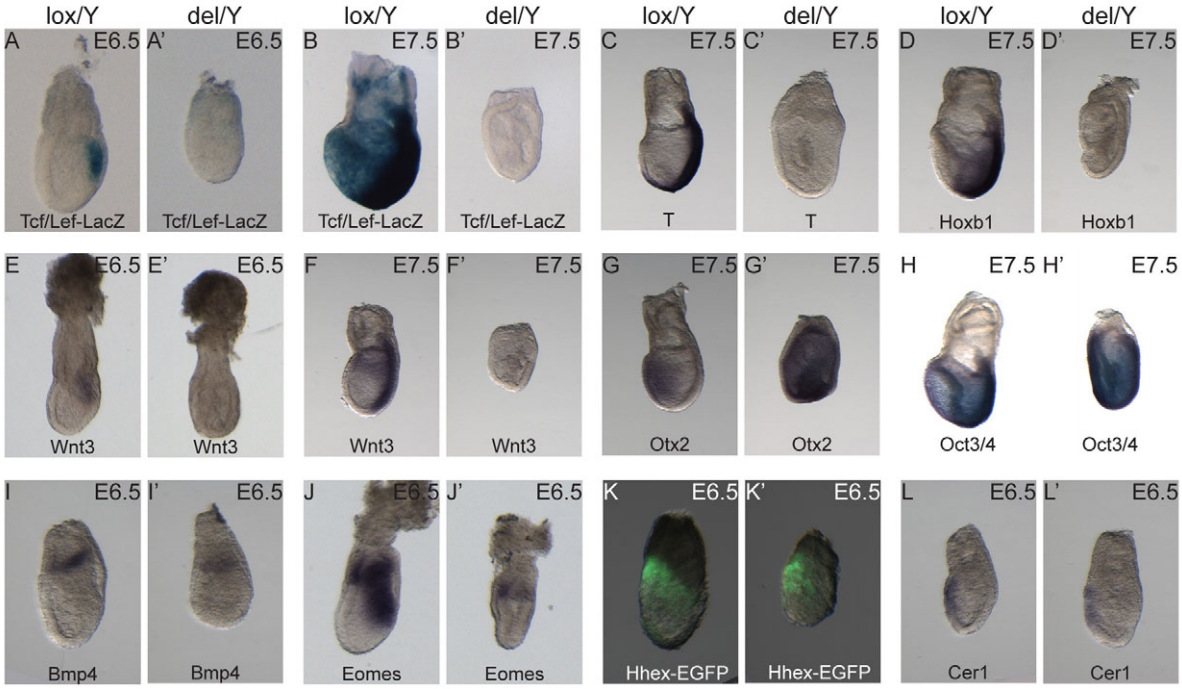

Fig. 2. *In situ* gene expression analysis of zygotic hemizygous *Porcn* mutants. Representative images of *Porcn*$^{lox/Y}$ (**A-L**) and *Porcn*$^{del/Y}$ (**A′-L′**) mouse embryos analyzed by *in situ* hybridization for marker genes or reporter gene expression. *Porcn* mutants fail to express the canonical Wnt signaling reporter *Tcf/Lef-lacZ* at E6.5 (A′) and E7.5 (B′), as well as the primitive streak marker brachyury (C′) and posterior cell fate marker *Hoxb1* (D′). The canonical Wnt signaling target and primitive streak-inducing *Wnt3* is not expressed in *Porcn* mutants at E6.5 (E′) and E7.5 (F′). By contrast, *Otx2* (G,G′) and *Oct3/4* (H,H′) are strongly expressed throughout the epiblast of mutant embryos at E7.5. The extra-embryonic region exhibits reduced expression of *Bmp4* (I,I′) and *Eomes* (J,J′). Expression of the anterior visceral endoderm (AVE) markers *Hhex-EGFP* (K,K′) and *Cer1* (L,L′) is unaffected in *Porcn* mutants.

expression is induced and maintained by *Bmp4* secreted from the extra-embryonic ectoderm (Ben-Haim et al., 2006; Miura et al., 2010), but it is also regulated by canonical Wnt signaling in an autoregulatory feedback loop (Tortelote et al., 2013) that maintains Wnt3 expression after its initiation at E5.75 (Rivera-Pérez and Magnuson, 2005). Consistent with autoregulation at and prior to E6.5 (Tortelote et al., 2013), we were unable to detect *Wnt3* transcript at E6.5 (n=11, Fig. 2E′) and E7.5 (n=6, Fig. 2F′) in *Porcn* mutant embryos. The expression of Wnt3-inducing *Bmp4* at E6.5 was slightly reduced but present in *Porcn* mutant embryos (n=3, Fig. 2I′). Consistent with all the above findings, *Eomes*, which is normally expressed in extra-embryonic ectoderm and mesoderm (n=24, Fig. 2J) (Russ et al., 2000), was absent in the embryonic region and reduced (n=3, Fig. 2J′) or absent (n=3) in the extra-embryonic region of *Porcn* mutant embryos at E6.5, suggesting that these embryos fail to maintain the extra-embryonic ectoderm, in addition to the gastrulation defect.

As canonical Wnt signaling has been implicated in anterior-posterior (AP) axis development in the mouse embryo (Huelsken et al., 2000; Morkel et al., 2003), we tested AP axis establishment in *Porcn* mutant embryos by examining the anterior localization of the anterior visceral endoderm (AVE) as visualized by the *Hhex-eGFP* transgene (Rodriguez et al., 2001). At E6.5, the majority of mutant embryos (n=7, Fig. 2K′) showed anterior localization of GFP$^+$ cells, suggesting that *Porcn*-mediated canonical Wnt signaling is not required for the anterior localization of the AVE. The remaining embryos showed distal localization (n=2) or strong reduction (n=3) of GFP expression, potentially indicating a developmental delay in *Porcn* mutant embryos. Confirming proper AVE localization, we detected *Cer1* (Shawlot et al., 1998) asymmetrically at E6.5 in the majority of both wild-type and mutant embryos (n=2/3 and n=3/4, Fig. 2L,L′) by *in situ* hybridization. The inability to detect *Cer1* in the remaining embryos is likely to be due to inefficient hybridization of this probe. These data suggest that *Porcn*-mediated Wnt signaling is not essential for the distal visceral endoderm (DVE) to AVE transition.

In summary, this analysis shows that *Porcn* is required for canonical Wnt signaling and gastrulation *in vivo*. This phenotype appears identical to that of zygotic *Wnt3* mutants (Liu et al., 1999), which also fail to initiate gastrulation. In contrast to *Porcn* mutants, epiblast-specific *Wnt3* mutants and *Porcn* null aggregation embryos initiate gastrulation but fail to maintain it (Barrow et al., 2007; Biechele et al., 2011; Tortelote et al., 2013). These observations suggest that VE-secreted Wnt3 is sufficient to induce the initial phases of gastrulation in *Porcn* or *Wnt3* mutant epiblast, as the extra-embryonic tissues are wild type in both settings.

Extra-embryonic deletion of *Porcn* produces a chorio-allantoic fusion defect and phenocopies *Wnt7b* mutants

To test whether Porcn function is necessary in extra-embryonic tissues, including the VE, we made use of female embryos with imprinted X chromosome inactivation (XCI) as well as a VE-specific Cre-mediated deletion of *Porcn*. As *Porcn* is an X-chromosomal gene it is subject to XCI in females (Barakat and Gribnau, 2012). Whereas XCI is random in the embryo proper, XCI is imprinted in the extra-embryonic trophoblast and primitive endoderm lineages, resulting in paternal-specific silencing (Takagi

and Sasaki, 1975). Heterozygous females carrying a mutant maternal *Porcn* allele [$Porcn^{del(Xm)/+(Xp)}$] are therefore mosaic in the embryo proper but have functionally mutant extra-embryonic tissues.

$Porcn^{lox/lox}$ females were crossed to males carrying a ubiquitously expressed *pCX-NLS-Cre* transgene (Belteki et al., 2005). Female embryos derived from this cross thus inherited a paternal wild-type *Porcn* allele (Xp) and a maternal floxed or zygotically deleted *Porcn* allele (Xm). Zygotically deleted $Porcn^{del(Xm)/+(Xp)}$ embryos were recovered up to E11.5 (Fig. 3A). These embryos could first be distinguished morphologically at E9.5 as they display a ball of allantoic tissue at the posterior end of the embryo, typical of failed chorio-allantoic fusion (Fig. 3B,C). $Porcn^{del/+}$ embryos fail to establish a functional umbilical cord and placenta, which are required to provide the embryo with nutrients and oxygen. Consistent with a lack of maternal nutrients and with embryonic hypoxia, $Porcn^{del/+}$ embryos failed to thrive compared with $Porcn^{lox/+}$ littermates (Fig. 3B,C). This phenotype is highly reminiscent of the *Wnt7b* (Parr et al., 2001) and integrin alpha 4 (*Itga4*) (Yang et al., 1995) mutant phenotypes. In contrast to a report that used an independent floxed *Porcn* and *Cre* allele to generate $Porcn^{del/+}$ embryos (Liu et al., 2012), we only rarely observed neural tube closure defects (n=2/33), which we attribute to a general developmental delay associated with the chorio-allantoic fusion defect.

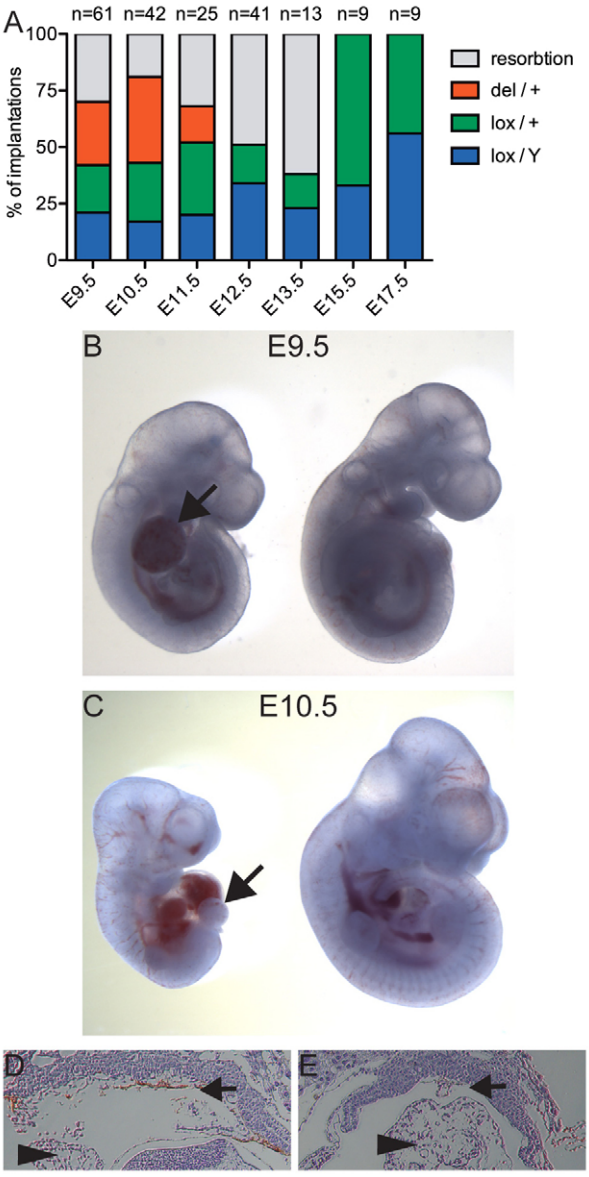

Fig. 3. Chorio-allantoic fusion defect in $Porcn^{del/+}$ female mouse embryos. (**A**) Quantification of genotypes from E9.5 to E17.5. $Porcn^{del/+}$ embryos are detected at Mendelian ratios (25%) at E9.5 and can be recovered up to E11.5. (**B**,**C**) $Porcn^{del/+}$ embryos (left) exhibit failure in chorio-allantoic fusion as indicated by a ball of allantoic tissue (arrow) at the posterior end of the embryo at E9.5 (B, n=17) and E10.5 (C, n=16) and a failure to thrive compared with control littermates (right). (**D**,**E**) The cause of this defect is the absence of integrin alpha 4 expression in the chorion (arrow) of $Porcn^{del/+}$ females (E) compared with control littermates (D). Allantoides are indicated by arrowheads.

The chorionic plate in the placenta at E9.5 did not significantly differ in size between mutants and control littermates (supplementary material Fig. S3A-E), suggesting that the defect was more likely to be in trophoblast cell function than cell proliferation. If $Porcn^{del/+}$ females fail to palmitoylate and secrete *Wnt7b* from the extra-embryonic chorion, this is likely to result in absence of the *Itga4* expression required for chorio-allantoic fusion, as seen in *Wnt7b* mutants (Parr et al., 2001). Consistent with this mechanism, Itga4 protein is undetectable in the chorion of $Porcn^{del/+}$ females at E8.5 by immunostaining (n=2, Fig. 3E), whereas it is readily detectable in $Porcn^{lox/+}$ females (n=3, Fig. 3D).

Our genetic approach, as well as the absence of a chorio-allantoic fusion defect in epiblast-specific heterozygotes (Barrott et al., 2011), clearly shows that *Porcn* is required in extra-embryonic tissues for chorio-allantoic fusion.

Porcn is not required in the visceral endoderm

The phenotype of the $Porcn^{del/+}$ embryos strongly suggests that the earliest extra-embryonic requirement for *Porcn*-mediated Wnt secretion is in the chorion at E8.5, leading to a lack of chorio-allantoic fusion and embryonic lethality by E11.5. However, several lines of evidence have suggested that Wnt signaling could also play an earlier extra-embryonic role in the VE. *Porcn* is expressed at E6.5 in the AVE (Biechele et al., 2011; Gonçalves et al., 2011) and *Wnt3* expression from the posterior VE is sufficient to induce gastrulation (Tortelote et al., 2013).

We investigated a potential requirement for *Porcn* in the VE by a direct approach that was not dependent on XCI, using the previously described VE-specific *Ttr::Cre* allele (Kwon and Hadjantonakis, 2009). Successful *Porcn* deletion by the *Ttr::Cre* allele was confirmed by PCR genotyping of embryos at E7.5 (supplementary material Fig. S4A). Despite successful deletion, $Porcn^{lox/Y}$; $Ttr::Cre^{+/tg}$ males and $Porcn^{lox/+}$; $Ttr::Cre^{+/tg}$ females were observed at the expected ratios at weaning age (supplementary material Fig. S4B) and were indistinguishable from Cre-negative $Porcn^{lox/Y}$ and $Porcn^{lox/+}$ littermates.

These results show that *Porcn* is not required in the Ttr::Cre^{+} VE and its derivatives for normal embryonic development and are consistent with the phenotype of zygotic $Porcn^{del/+}$ females. In combination with the results from zygotic and epiblast-specific *Porcn* and *Wnt3* mutants (Barrow et al., 2007; Biechele et al., 2011; Tortelote et al., 2013), these results show that Wnt3 secreted from wild-type VE is sufficient to induce gastrulation in mutant epiblast but may not be required for the induction of gastrulation in wild-type epiblast or can function in the absence of Porcn in the VE.

Porcn-mediated Wnt signaling is not required prior to gastrulation

All of our studies of Porcn up to this point showed that Porcn function is not required prior to gastrulation. Although several Wnt ligands are expressed in the preimplantation blastocyst (Kemp et al., 2005), no Wnt pathway mutants described to date exhibit defects prior to the egg cylinder stage (van Amerongen and Berns, 2006). However, recent data suggest that Porcn-mediated Wnt signaling is necessary for the maintenance of pluripotent ESCs *in vitro* (ten Berge et al., 2011; Habib et al., 2013). As ESCs are considered to be the *in vitro* equivalent of the epiblast progenitors in the blastocyst, we determined whether Porcn-dependent Wnt secretion is also required for development to the blastocyst stage and early postimplantation stages *in vivo*.

Whereas *Porcn* transcript is detectable at increasing levels from the 2-cell to the morula stage and at low levels in blastocysts (Casanova et al., 2012; Hamatani et al., 2004; Xie et al., 2010), it is undetectable in oocytes (Macfarlan et al., 2012; Posfai et al., 2012), suggesting that rescue by maternal protein is unlikely to explain the absence of any early embryo defects in *Porcn* zygotic mutants. In order to test this more directly, we generated $Porcn^{lox/lox}$; $Zp3\text{-}Cre^{+/tg}$ females that delete *Porcn* in the developing oocytes (Lewandoski et al., 1997) and crossed them to wild-type males carrying an X-linked *EGFP* transgene (Hadjantonakis et al., 1998). The transgene allowed us to sex and thus genotype the resulting embryos: $Porcn^{del/+}$ females are fluorescently labeled by the X-linked *GFP* transgene, whereas maternal zygotic $Porcn^{del/Y}$ ($mzPorcn^{del/Y}$) male embryos are not.

Hemizygous mutant male and heterozygous female embryos implanted successfully with no apparent deviation from the expected ratio (1:1) when recovered at E6.5 and E7.5 (Fig. 4E). The recovered mutant male embryos failed to gastrulate and morphologically resembled zygotic *Porcn* mutants. This observation was confirmed by *in situ* hybridization for *Bmp4*, *Cer1* and *Otx2* (Fig. 4A-C′). Furthermore, expression of the canonical Wnt signaling target *Axin2* (Jho et al., 2002) was undetectable in $mzPorcn^{del/Y}$ embryos (n=5, Fig. 4D) but apparent in $Porcn^{del/+}$ littermates at E7.5 (n=4, Fig. 4D′). These results support the conclusion that maternal Porcn expression and early Porcn-dependent Wnt secretion are not crucial for early development.

Blastocyst-secreted Wnt ligands have been proposed to play a role in implantation, as Wnt-coated beads are sufficient to activate the canonical Wnt signaling pathway in the luminal epithelium of the uterus (Mohamed et al., 2005). Further, Wnt pathway inhibition by Sfrp2 reduces implantation rates (Mohamed et al., 2005), suggesting that Wnt signaling from the blastocyst might be required for implantation. The fact that $mzPorcn^{del/Y}$ embryos successfully implanted and developed to E7.5 would tend to argue against a key role for Porcn-dependent blastocyst-derived Wnts in promoting implantation. However, we could not rule out the possibility that implantation was rescued by Wnt signals produced by heterozygous littermates present in the same uterus.

To test this possibility, we separated blastocysts from the above cross at E3.5 by sex and transferred them separately into surrogate mothers. Dissection at E7.5 revealed that male mutant embryos and female heterozygous embryos implanted successfully at similar frequencies of 53% (n=17) and 60% (n=10), respectively (Fig. 4F). These results substantiate our finding that Porcn-dependent Wnt ligands secreted from the embryo are not required for implantation.

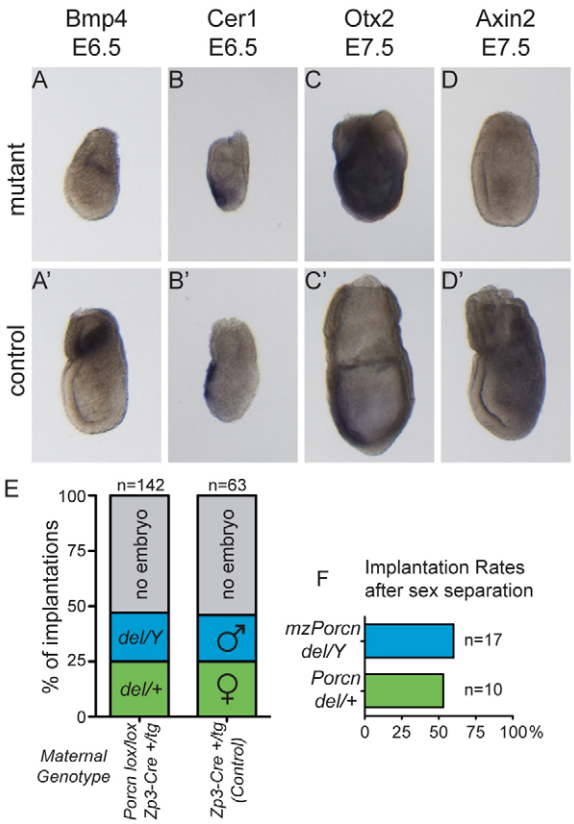

Fig. 4. Gastrulation defect in maternal zygotic *Porcn* mutants. (**A-D′**) Representative images of maternal zygotic *Porcn* mutant (A-D) and control (A′-D′) mouse embryos analyzed by *in situ* hybridization. (A-B′) At E6.5, mutant embryos show normal localization of *Bmp4* (A) and *Cer1* (B) expression. At E7.5, *Otx2* is expressed ectopically throughout the epiblast of *Porcn* mutants (C), whereas the canonical Wnt signaling target *Axin2* is undetectable (D). (**E**) Dissection of litters from $Porcn^{lox/lox}$; $Zp3\text{-}Cre^{+/tg}$ females at E7.5 revealed the expected 1:1 ratio of the two embryonic genotypes. The presence of empty decidua (50% of implantations) in the mutant litters (E) was also observed in control crosses using $Zp3\text{-}Cre^{+/tg}$ control females (E), suggesting that this lethality is due to the *Zp3-Cre* transgene. (**F**) After sex-separated transfer into wild-type surrogates at E3.5, both embryonic genotypes ($mzPorcn^{del/Y}$ and $Porcn^{del/+}$) showed similar implantation rates at E7.5.

Normal cell fate establishment in *Porcn* mutant blastocysts

As maternal zygotic *Porcn* mutant embryos develop successfully to pre-gastrulation stages, preimplantation development cannot be severely affected by loss of Porcn-dependent Wnt signaling, consistent with the findings of published studies in which canonical Wnt signaling was manipulated in preimplantation development (Haegel et al., 1995; Kemler et al., 2004; Xie et al., 2008). However, mild effects on lineage allocation would not necessarily be incompatible with normal implantation (Kang et al., 2013).

To determine whether there was any defect in lineage segregation, we immunostained maternal/zygotic *Porcn* mutant and control blastocysts for the lineage-specific markers Nanog (epiblast), Gata6 (primitive endoderm) and Cdx2 (trophoblast). We also compared results with embryos carrying a stabilized allele of β-catenin (Harada et al., 1999) that activates canonical Wnt signaling. All embryos were recovered at E3.5 and cultured to E4.5. All three lineages were present and appropriately located in embryos of all

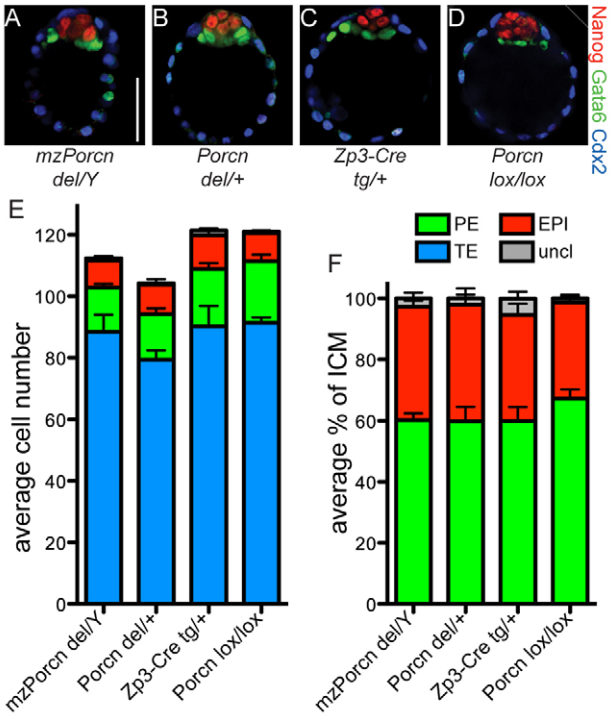

Fig. 5. Preimplantation development is unperturbed in *Porcn* mutants. The *Porcn* floxed allele was backcrossed to the C57BL/6J background (>F5) and deleted using the *Tg(Zp3-cre)93Knw/J* transgene, avoiding the artifactual lethality of the *Tg(Zp3-Cre)3Mrt* transgene observed in previous experiments (see Fig. 4E). (**A-D**) Representative confocal sections of E4.5 blastocysts immunostained for Nanog (epiblast), Gata6 (primitive endoderm) and Cdx2 (trophoblast). (**E**) No significant differences were observed in the quantification of cell fates between maternal zygotic *Porcn* mutants and control genotypes (chi-square test, P=0.5). The average cell number/embryo for each cell fate is shown (n=5 embryos/genotype). (**F**) Normalized cell fate distributions within the inner cell mass (ICM). TE, trophectoderm; PE, primitive endoderm; EPI, epiblast; uncl, unclassified cells. Error bars indicate s.e.m.

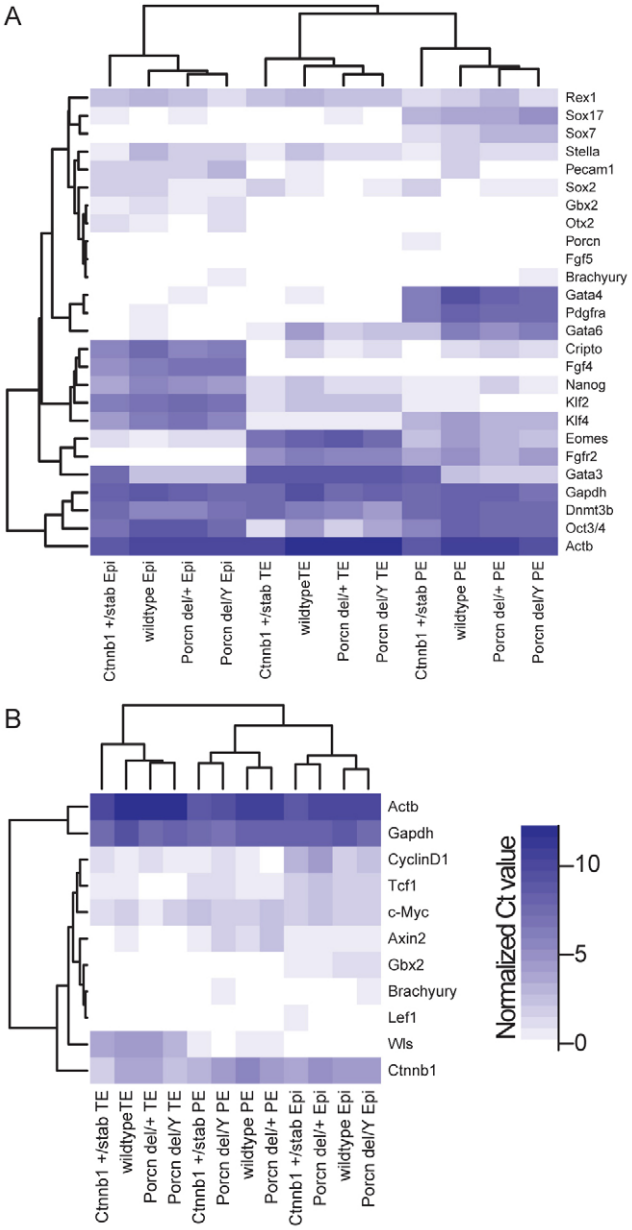

Fig. 6. Genetic manipulation of Wnt signaling has no detectable effect on blastocyst lineage marker gene expression. (**A**) Heat map of cell fate marker gene expression in E4.5 mouse blastocysts. Single-cell gene expression levels in *Porcn*$^{del/Y}$ (n=35), *Porcn*$^{del/+}$ (n=49), *Porcn*$^{+/+}$ (n=93) and *Ctnnb1*$^{+/del\,ex3}$ (n=180) embryos were determined using the BioMark system and assigned to one of the three lineages (TE, PE, Epi) based on marker genes. We were unable to detect major differences in median gene expression levels between genotypes within each cell lineage as shown by unsupervised hierarchical clustering of the samples. (**B**) Heat map of direct canonical Wnt target gene expression in E4.5 blastocysts. We were unable to detect significant differences in Wnt target gene expression levels between *Porcn* mutant, wild-type and *Ctnnb1*-stabilized embryos within each cell lineage as shown by unsupervised hierarchical clustering of the samples. Transcript levels of *Ctnnb1* are unaffected across all genotypes. *Actb* and *Gapdh* are not Wnt signaling targets but served as housekeeping controls. TE, trophectoderm; PE, primitive endoderm; Epi, epiblast.

genotypes (Fig. 5A-D; supplementary material Fig. S5A-C). Quantification of cell numbers for each lineage revealed that both *Porcn*$^{del/Y}$ and *Porcn*$^{del/+}$ had normal cell numbers and cell fate distributions compared with control embryos (Fig. 5E; chi-square analysis, P=0.5). Activating the downstream canonical Wnt pathway by stabilization of β-catenin increased the total number of ICM cells, approaching statistical significance (supplementary material Fig. S5D; chi-square analysis, P=0.051), but the ratio of Nanog^{+} epiblast to Gata6^{+} primitive endoderm cells remained similar to that of wild-type or *Porcn* mutant embryos (supplementary material Fig. S5E). The number of outer trophectoderm cells remained similar to that of control embryos (supplementary material Fig. S5D). These *in vivo* findings show that Porcn-dependent Wnt signaling is not necessary for ICM maintenance, but activated Wnt signaling is sufficient to increase the number of ICM cells.

We also assessed whether ablation of Porcn-dependent Wnt signaling resulted in a molecular phenotype at the transcriptional level. We dissociated E4.5 blastocysts and performed quantitative gene expression analysis using the BioMark system (Rugg-Gunn et al., 2012). Gene expression in single cells was analyzed for a panel of cell fate marker genes, as well as direct canonical Wnt signaling targets (supplementary material Tables S2 and S4). Unsupervised clustering of gene expression levels for all genes analyzed revealed that all three cell fates of the blastocyst are established in the *Porcn* mutant as well as in β-catenin-stabilized embryos and show highly

similar gene expression profiles to control embryos (Fig. 6A). Importantly, the main driver of clustering is cell fate and not genotype. The canonical Wnt target genes assessed showed lineage-specific expression patterns (Fig. 6B) but no significant changes in expression levels upon genetic inactivation of *Porcn* or downstream activation of the canonical Wnt pathway, suggesting that the canonical Wnt pathway is not fully functional at this stage of development. Together, these data confirm that the three cell fates of the blastocyst are established independently of Porcn and canonical Wnt pathway manipulation.

Porcn is not essential for the maintenance of pluripotency *in vitro* and *in vivo*

To complement these *in vivo* studies, we investigated whether pluripotency *in vitro* is dependent on Porcn-mediated canonical Wnt signaling. We cultured both $Porcn^{lox/Y}$ and $Porcn^{del/Y}$ ESCs in feeder-free, chemically defined conditions in chemically defined conditions including LIF and the Mek inhibitor PD0325901 (Nichols and Ying, 2006), as well as the Gsk3 inhibitor CHIR99021 or the Porcn inhibitor IWP2. Surface marker expression was analyzed for ESC and epiblast stem cell (EpiSC) markers by flow cytometry (Rugg-Gunn et al., 2012). Cells in all conditions maintained similarly high levels of the ESC markers CD31 (Pecam1 – Mouse Genome Informatics) (supplementary material Fig. S6A-F) and CD81 (supplementary material Fig. S6I), and low levels of the EpiSC marker CD40 (supplementary material Fig. S6A-F). These data suggest that Porcn-mediated Wnt signaling is not required for the maintenance of pluripotency in ESCs.

In contrast to cultured ESCs *in vitro*, pluripotency in the embryo is a transient state from E3.5 to E5.5. It is thus possible that Porcn-mediated Wnt signaling *in vivo* is only required for prolonged maintenance of epiblast progenitor cells during diapause, similar to components of the LIF receptor complex (Nichols et al., 2001). To test this, we delayed implantation of *Porcn* mutant embryos *in vivo* for 6 days by chemically inducing diapause to EDG10 (equivalent days of gestation) (Hunter, 1999). Mutant preimplantation embryos flushed at EDG10 had normal morphology (supplementary material Fig. S6J,K) and were transferred into surrogates where they successfully implanted (n=9/10) and displayed the characteristic gastrulation phenotype at E7.5 (according to surrogate pregnancy). These data substantiate that embryonic *Porcn* deletion has no functional effects on preimplantation development *in vivo*.

DISCUSSION

Wnt ligands have been shown to play numerous and redundant roles in mammalian embryonic development. As all Wnts are dependent on Porcn function for their secretion (Najdi et al., 2012), Porcn represents a bottleneck for all pathways activated by Wnt ligands. In this study, we have used zygotic and tissue-specific ablations of *Porcn* to ablate Wnt ligand secretion in embryonic development. Using this approach, we have determined the earliest requirements for embryonic and extra-embryonic Porcn-dependent Wnt ligand secretion.

Although epiblast-specific *Porcn* mutants have been reported (Barrott et al., 2011; Biechele et al., 2011), the phenotype of zygotic inactivation of *Porcn* has not been described in any detail (Liu et al., 2012). We show here that zygotic *Porcn* mutants fail to gastrulate and remain in an $Oct3/4^{+}$ $Otx2^{+}$ epiblast-like state, similar to *Porcn* epiblast-specific mutants. This not only phenocopies *Wnt3* mutants (Liu et al., 1999), but also a group of 'canonical Wnt null' phenotypes, such as *Wls* (Fu et al., 2009), *Mesd1* (*Mesdc1* – Mouse Genome Informatics) (Hsieh et al., 2003), *Lrp5/6* compound mutants (Kelly et al., 2004), and epiblast-specific *Ctnnb1* mutants (Rudloff and Kemler, 2012). Strikingly, the phenotype of the downstream effector *Ctnnb1* differs slightly (Haegel et al., 1995; Huelsken et al., 2000; Morkel et al., 2003). Whereas all 'canonical Wnt null' mutants show a proper DVE to AVE transition, *Ctnnb1* mutants fail to establish the AVE signaling center that is indicative of AP axis formation. Our data support the notion that this phenotypic discrepancy reflects a Porcn/Wnt-independent function of β-catenin (Morkel et al., 2003).

Although *in vitro* data suggest that Porcn acts on all Wnt ligands (Najdi et al., 2012), it remains unclear whether Porcn affects both canonical and non-canonical Wnt signaling *in vivo* (Chen et al., 2012; Galli and Burrus, 2011). Owing to the early lethality of zygotic *Porcn* mutants, we were not able to assess later effects on convergent extension or planar cell polarity (PCP), which have been associated with defects in non-canonical Wnt signaling (Andre et al., 2012; Gao et al., 2011; Ho et al., 2012). The pre-gastrulation phenotype of mutants for the PCP component *Mpk1* (*Prickle1* – Mouse Genome Informatics) shows similarities to that of *Ctnnb1*, but has been attributed to its PCP-independent function in apical-basal polarity (Huelsken et al., 2000; Tao et al., 2009).

In contrast to zygotic *Porcn* mutants, data from epiblast-specific mutants show a failure to induce the primitive streak marker brachyury at E6.5 (Barrott et al., 2011), but some residual Wnt signaling response and delayed induction of brachyury at E7.5 (Biechele et al., 2011). This phenotypic discrepancy suggests that the VE is a transient source of Wnt ligands at E7.5. This finding is supported by a recent study showing that Wnt3 secreted from the VE is sufficient to induce, but not maintain, gastrulation in epiblast-specific *Wnt3* mutants (Barrow et al., 2007; Tortelote et al., 2013). In order to determine whether Porcn-dependent Wnt secretion from the VE is also necessary, we generated embryos with *Porcn* functionally mutant extra-embryonic tissues based on imprinted XCI. Surprisingly, these embryos were embryonic lethal due to a defect in chorio-allantoic fusion, similar to *Wnt7b* mutants (Parr et al., 2001). As Wnt7b-mediated chorio-allantoic fusion occurs ~1 day after *Wnt3*-induced gastrulation, we conclude that Porcn-dependent Wnt secretion from the VE is not necessary for gastrulation, but that Porcn-dependent Wnt signaling is required from an extra-embryonic source for the development of the chorio-allantoic placenta. In contrast to mice, human focal dermal hypoplasia (FDH) patients can inherit a mutant X-chromosomal *PORCN* allele from either parent (Grzeschik et al., 2007). This discrepancy in phenotypes is most likely due to the lack of stringency in imprinted extra-embryonic XCI in humans (Zeng and Yankowitz, 2003).

In order to validate further that there is no role for *Porcn*-mediated Wnt signaling in the VE, we generated VE-specific *Porcn* mutants using the *Ttr::Cre* allele (Kwon and Hadjantonakis, 2009). In keeping with data from $Porcn^{del/+}$ females, VE-specific deletion had no effect on embryonic development in males or females. Thus, multiple lines of evidence suggest that Porcn-mediated Wnt secretion from the VE and its derivatives, despite being sufficient (Tortelote et al., 2013), is not necessary for the induction of gastrulation or for further development to adulthood.

The phenotype of zygotic *Porcn* mutant embryos shows that Porcn-dependent Wnt secretion is not necessary prior to gastrulation (E6.5). This is in contrast to studies suggesting functions for embryo-secreted Wnt ligands in implantation (Mohamed et al., 2005). We have investigated these questions by oocyte-specific deletion of *Porcn* (de Vries et al., 2000; Lewandoski et al., 1997), thereby eliminating the possibility of maternal rescue. Using this

approach, we were able to show that maternal zygotic *Porcn* mutant embryos display no implantation defects even in the absence of heterozygous littermates. These data clearly show that *Porcn*-mediated Wnt signaling from the embryo is not required for implantation or preimplantation development. They further support a model in which an unknown factor secreted from blastocysts is sufficient to induce Wnt ligand expression in the uterine epithelium (Mohamed et al., 2005).

Data obtained from *in vitro* studies in ESCs show that *Porcn*-mediated Wnt signaling and Wnt3a protein contribute to maintaining ESCs in a pluripotent state (ten Berge et al., 2011; Habib et al., 2013). Further, activation of canonical Wnt signaling by Gsk3 inhibition allows efficient derivation and maintenance of ESCs (Ying et al., 2008). As ESCs are derived from the epiblast of blastocysts, it has been suggested that Wnt signaling is also required in the ICM *in vivo*. The fact that maternal zygotic *Porcn* mutants, as well as canonical Wnt receptor and *Ctnnb1* mutants (Kelly et al., 2004; Rudloff and Kemler, 2012; Valenta et al., 2011), develop to gastrulation stages shows, however, that Porcn-dependent canonical Wnt signaling is not strictly required for the maintenance of the ICM *in vivo*. Further, in contrast to LIF signaling (Nichols et al., 2001), we show that Porcn-mediated Wnt signaling is not required for the prolonged maintenance of epiblast during diapause *in vivo*.

Unlike studies that have proposed a role for Porcn-mediated Wnt signaling in the maintenance of pluripotent ESCs (ten Berge et al., 2011; Habib et al., 2013), we have been unable to reveal such an effect in our studies, potentially owing to the inclusion of Mek inhibitor in the media, which might reduce the dependence on Wnt signaling. Consistent with other recent studies (del Valle et al., 2013; Faunes et al., 2013; Rudloff and Kemler, 2012), however, our data do not preclude a non-transcriptional role for β-catenin in the maintenance of pluripotency *in vitro*.

To determine whether there was a more subtle, non-lethal effect of *Porcn* ablation *in vivo*, similar to heterozygous *Fgf4* ablation (Kang et al., 2013), we investigated blastocysts with maternal and zygotic deletion of *Porcn*, or activated Wnt signaling (*Ctnnb1*$^{del\,ex3}$). Whereas genetic activation of the canonical Wnt signaling pathway was sufficient to increase the number of ICM cells of the blastocyst, ablation of *Porcn* had no effect on cell numbers or cell fate decisions in preimplantation development. At a molecular level, the gene expression profiles for numerous cell fate marker genes remain highly similar between wild-type embryos and embryos with genetic ablation of *Porcn* or activation of canonical Wnt signaling activity. Our results clearly show that *Porcn*, and thus Porcn-dependent Wnt ligands, are not required for preimplantation development.

Although several direct canonical Wnt signaling target genes exhibit lineage-specific expression patterns in the blastocyst, no target gene was significantly responsive to genetic activation or inactivation of the Porcn-dependent Wnt signaling pathway. These data suggest that the Porcn-mediated Wnt signaling response is inhibited or dampened in preimplantation development. This dampening might be mediated by the Hippo pathway, which is actively involved in cell fate decisions in the blastocyst (Nishioka et al., 2009) and has recently been shown to be able to inhibit canonical Wnt signaling by retaining β-catenin in the cytoplasm (Imajo et al., 2012).

In summary, we have conclusively shown that *Porcn* is first required in embryonic tissues for the induction of gastrulation mediated by *Wnt3*. In extra-embryonic tissues, *Porcn* function is first required for chorio-allantoic fusion, probably for the secretion of chorionic *Wnt7b*. However, Porcn-mediated Wnt signaling is not required for implantation or preimplantation development. In combination with published chemical and genetic modifiers of Wnt signaling, this floxed allele will be a useful tool to investigate Porcn function, Wnt ligand secretion and redundancy both *in vitro* and *in vivo*.

Acknowledgements
We thank Jorge Cabezas for assistance with mouse husbandry; Malgosia Kownacka and Jodi Garner for assistance with tissue culture; Angela McDonald for flow cytometry; Andres Nieto for Fluidigm assistance; Oliver Tam for discussions; the Toronto Centre for Phenogenomics (TCP) and specifically the TCP Transgenic Core Facility for excellent support in mouse generation and embryo transfers; Dr Kat Hadjantonakis for sharing of the *Ttr-Cre* transgenic mouse line; and the Samuel Lunenfeld Research Institute's CMHD Pathology Core for Histology for their technical services.

Funding
This work was supported by the March of Dimes Foundation [research grant 6-FY08-315] and the Canadian Institutes of Health Research (CIHR) [grant #FRN13426].

Competing interests statement
The authors declare no competing financial interests.

Author contributions
S.B. designed and performed experiments, contributed to data analysis and wrote the manuscript. K.C. and F.L. performed experiments and contributed to data analysis. B.J.C. performed data analysis and contributed to experimental design. J.R. contributed to experimental design and data analysis and wrote the manuscript. All authors read and approved the final manuscript.

Supplementary material
Supplementary material available online at
http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.094458/-/DC1

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