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

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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
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 chorioallantoic 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 ampiclon. 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, CTGTAAACAAGACATGACCTTCA; PorcnRecR1, TAACTAGGACCTTGGGATAGGAT; and PorcnRecR3, GTTCTGCC- TCCCCTAACCATAAC.

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 transgens; D4/XEGFP [Tg(FGFP)xNagy] (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)Rhe] (Rodriguez et al., 2001), Zp3-Cre [Tg(Zp3-Cre)3Mr] (Lewandoski et al., 1997), Tcf/Lef-cre [Tg(Tcf/cre)1Had] (Kwon and Hadjantonakis, 2009) and Ctnnb1floxed(ex3) (Ctnnb1mis(sh) (Harada et al., 1999). For blastocyst studies, the incipient gonad congenic (F5) Porcn floxed (C57BL/6J) allele was deleted using a different Zp3-Cre allele [Tg(Zp3-cro)95Kna/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<sup>mut</sup>=<sup>mat</sup>).

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 α4 immunohistochemistry was performed as described (Daane et al., 2011).

Sex-separated and diapause pregnancies
Embryos were generated by natural mating of Porcn<sup>lox<sup>loxx</sup></sup>, Zp3-Cre<sup>(Mrt)<sup>+<sup>deg</sup></sup></sup> females to XEGFP<sup>neo<sup>deg</sup></sup> males. Embryos were harvested at E3.5 [or EDG10 after diapause induction (Hunter, 1999)], sexed based on EGFFP 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<sub>2</sub> 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 Axiosvert 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 trophoblast 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-Nis-Cre<sup>neo</sup>; Ctnnb1<sup>1<sup>del</sup>ex<sup>3</sup></sup>) 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<sup>del<sup>3</sup></sup>, n=35; Porcn<sup>del<sup>1</sup></sup>, n=49; Porcn<sup>2<sup>del</sup></sup>, n=93; Ctnnb1<sup>1<sup>del</sup>ex<sup>3</sup></sup>, 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, GGCTGCTTCTTACCATCCTGC; PorcnEx2-4R, TGTCCACCAGTGACATTCA; PorcnEx4-9F, GTGACATTGGTCGGCGA; and PorcnEx4-9R, ACGTCTAGGTCC- CATTCCAG. Autocrine Tcf/Lef-luciferase assays were performed as described (Biechele et al., 2011)

For flow cytometric analysis, a single-cell suspension of Porcn<sup>1<sup>loc<sup>Y</sup></sup></sup> or Porcn<sup>2<sup>loc<sup>Y</sup></sup></sup> (H4D4) or Porcn<sup>1<sup>loc<sup>Y</sup></sup></sup> (H4D4F9) ESCs was plated onto gelatin-serum-coated plates at 10,000 cells/cm<sup>2</sup> 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<sup>2</sup>. 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<sup>lox<sup>loxx</sup></sup>; 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-
deleted ESCs (Porcn<del>del</del>) 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>del</del> 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<del>del</del> ESCs (Fig. 1H). By contrast, Porcn<del>del</del> 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>del</del>) 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>del</del> embryos continued to express the pluripotency marker Otx3/4 (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
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-Perez 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 Cerl (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 Cerl 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.

**Fig. 2. In situ gene expression analysis of zygotic hemizygous Porcn mutants.** Representative images of Porcnlox/lox (A-L) and Porcndel/del (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 Cerl (L,L') is unaffected in Porcn mutants.
Porcn roles in mouse embryos

and Sasaki, 1975). Heterozygous females carrying a mutant maternal Porcn allele [Porcn<sup>del(Xm)/+</sup>] are therefore mosaic in the embryo proper but have functionally mutant extra-embryonic tissues.

Porcn<sup>lox/lox</sup> 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<sup>del(Xm)/+/Xp</sup> 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<sup>del/+</sup> 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<sup>del/+</sup> embryos failed to thrive compared with Porcn<sup>lox/+</sup> 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<sup>del/+</sup> 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.

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<sup>del/+</sup> 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<sup>del/+</sup> females at E8.5 by immunostaining (n=2, Fig. 3E), whereas it is readily detectable in Porcn<sup>lox/+</sup> 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<sup>del/+</sup> 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<sup>lox/−</sup>; Ttr::Cre<sup>−/−</sup> males and Porcn<sup>lox/−</sup>; Ttr::Cre<sup>−/−</sup> females were observed at the expected ratios at weaning age (supplementary material Fig. S4B) and were indistinguishable from Cre-negative Porcn<sup>lox/−</sup> and Porcn<sup>lox/−</sup> littermates.

These results show that Porcn is not required in the Ttr::Cre<sup>+</sup> VE and its derivatives for normal embryonic development and are consistent with the phenotype of zygotic Porcn<sup>del/+</sup> 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.

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Fig. 3. Chorio-allantoic fusion defect in Porcn<sup>del/+</sup> female mouse embryos. (A) Quantification of genotypes from E9.5 to E17.5. Porcn<sup>del/+</sup> embryos are detected at Mendelian ratios (25%) at E9.5 and can be recovered up to E11.5. (B,C) Porcn<sup>del/+</sup> 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) Allantoides are indicated by arrowheads.
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 Porcnlox/lox; Zp3-Cre 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 genotype the resulting embryos: Porcndel/– females are fluorescently labeled by the X-linked GFP transgene, whereas maternal zygotic Porcn–/– (mzPorcn–/–) 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–/– embryos (n=5, Fig. 4D) but apparent in Porcn–/– 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–/– 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.

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 (Haegele et al., 1995; Kessler 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
genotypes (Fig. 5A-D; supplementary material Fig. S5A-C). Quantification of cell numbers for each lineage revealed that both Porcndel/Y and Porcndel/+ 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 \(\beta\)-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 trophoderm 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 \(\beta\)-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<sup>locl</sup> and Porcn<sup>del/del</sup> 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), Mesp1 (Mandel – 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 (Hagel 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 Burus, 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 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<sup>Cre</sup> allele (Kwon and Hadjantonakis, 2009). In keeping with data from Porcn<sup>del/del</sup> 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 maternal Porcn deletion in mouse embryos does not result in implantation defects, as well as canonical Wnt signaling required in the ICM of blastocysts, it has been suggested that 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 Fgfd4 ablation (Kang et al., 2013), we investigated blastocysts with maternal and zygotic deletion of Porcn, or activated Wnt signaling (Ctnnb1del/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 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.

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