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Wnt/β-catenin signalling regulates *Sox17* expression and is essential for organizer and endoderm formation in the mouse

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SUMMARY

Several signalling cascades are implicated in the formation and patterning of the three principal germ layers, but their precise temporal-spatial mode of action in progenitor populations remains undefined. We have used conditional gene deletion of mouse β-catenin in Sox17-positive embryonic and extra-embryonic endoderm as well as vascular endothelial progenitors to address the function of canonical Wnt signalling in cell lineage formation and patterning. Conditional mutants fail to form anterior brain structures and exhibit posterior body axis truncations, whereas initial blood vessel formation appears normal. Tetraploid rescue experiments reveal that lack of β-catenin in the anterior visceral endoderm results in defects in head organizer formation. Sox17 lineage tracing in the definitive endoderm (DE) shows a cell-autonomous requirement for β-catenin in midgut and hindgut formation. Surprisingly, wild-type posterior visceral endoderm (PVE) in midgut- and hindgut-deficient tetraploid chimera rescues the posterior body axis truncation, indicating that the PVE is important for tail organizer formation. Upon loss of β-catenin in the visceral endoderm and DE lineages, but not in the vascular endothelial lineage, *Sox17* expression is not maintained, suggesting downstream regulation by canonical Wnt signalling. Strikingly, Tcf4/β-catenin transactivation complexes accumulated on *Sox17* cis-regulatory elements specifically upon endoderm induction in an embryonic stem cell differentiation system. Together, these results indicate that the Wnt/β-catenin signalling pathway regulates *Sox17* expression for visceral endoderm pattering and DE formation and provide the first functional evidence that the PVE is necessary for gastrula organizer gene induction and posterior axis development.

KEY WORDS: Sox17, Wnt/β-catenin signalling, Endoderm, Gastrula organizer

INTRODUCTION

In the last two decades the molecular analysis of axis induction and embryonic patterning has provided a blueprint for body axis formation and organizer function in several species, including amphibians, chicken, zebrafish and mouse (Arnold and Robertson, 2009; De Robertis et al., 2000; Niehrs, 2004; Nowotschin and Hadjantonakis, 2010). In the mouse, the anterior-posterior (A-P) axis is firmly established when the anterior visceral endoderm (AVE) is formed on the future anterior side of the embryo before gastrulation commences at embryonic day (E) 6.5. At this stage, the embryo resembles an egg cylinder-shaped epiblast that is surrounded by extra-embryonic visceral endoderm (exVE), which constitutes the endodermal component of the yolk sac, and by extra-embryonic ectoderm (ExE) that gives rise to the placenta. The pluripotent epiblast cells give rise to all embryonic lineages, namely ectoderm, mesoderm and definitive endoderm (DE), during gastrulation at E6.5-7.5.

At E5.5, Bmp4 from the ExE induces autoregulatory Nodal signalling that spreads through the epiblast in a proximal to distal direction to induce the Nodal inhibitor Lefty1 and the Wnt inhibitors Dkk1 and Cerl in the distal visceral endoderm (DVE) (Brennan et al., 2001). Migration of the DVE cells to the anterior side of the epiblast and the formation of the AVE converts the proximal-distal (P-D) axis into the A-P axis between E5.5 and E6.5 (Takaoka et al., 2011; Yamamoto et al., 2004). The AVE cells express genes such as *Dkk1* (Mukhopadhyay et al., 2001), *Hex* (Martinez-Barbera et al., 2000), *Hesx1* (Hermesz et al., 1996; Thomas and Beddington, 1996), *Lim1* (*Lhx1*) (Barnes et al., 1994; Shawlot and Behringer, 1995) and *Otx2* (Ang et al., 1994; Simeone et al., 1992; Simeone et al., 1993), which are involved in anterior neuroectoderm induction. Mutations in these genes specifically result in anterior head truncations, indicating that the AVE displays head organizer function (Beddington and Robertson, 1998).

Besides this function, the AVE also expresses inhibitors of the Wnt/β-catenin and Nodal/TGFβ signalling pathways, which restricts the activity of these pathways to the posterior side of the embryo. Here, *Wnt3* is first expressed in the posterior visceral endoderm (PVE) beginning at E5.5 (Rivera-Pérez and Magnuson, 2005) and is subsequently induced in the proximal and posterior epiblast via a Nodal-dependent Bmp4 activation at E5.5-6.5 (Ben-Haim et al., 2006). The restriction of Nodal/TGFβ and Wnt/β-catenin signalling to the posterior epiblast allows the formation of the gastrula organizer, the formation of the primitive streak (PS), and specifies the mesoderm and DE in a dose-dependent manner at E6.5-7.5 (Arnold and Robertson, 2009). The pre-gastrula stage embryo is patterned before organizer genes, such as *Gsc*, *Foxa2*, *Chrd* and *Nog*, are induced in the posterior epiblast region during gastrulation (Beddington and Robertson, 1998; Kinder et al., 2001; Takaoka et al., 2011; Thomas and Beddington, 1996). It has long been known that the PVE covers the posterior PS region where the gastrula organizer is induced (Tam and Beddington, 1992); however, whether it is essential for gastrula organizer gene induction remains a long-standing question (Beddington and Robertson, 1999; Liu et al., 1999; Rivera-Pérez and Magnuson, 2005; Tam and Behringer, 1997).

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The gastrula organizer consists of a dynamic population of cells and can be subdivided into an early (EGO), mid- (MGO) and late (LGO) gastrula organizer, which possess head-, trunk- and tail-organising activity, respectively (Kinder et al., 2001). These organizer regions are mapped to different progenitor cells of the epiblast and give rise to different cell fates. The EGO emerges from Gsc^+ and $Foxa2^+$ epiblast cells that give rise to anterior definitive endoderm (ADE), prechordal mesoderm and axial mesoderm at E6.5 (Burtscher and Lickert, 2009; Kinder et al., 2001). Together with the AVE, the ADE and prechordal mesoderm constitute the head organizer (Arkell and Tam, 2012). The MGO and LGO are marked in the posterior epiblast by *Foxa2*, *Gsc*, *Chrd* and *Nog* expression at mid-streak (E7.0) to early bud stage (E7.75) and give rise to anterior endoderm, prechordal mesoderm, notochord and node. These tissues have the ability to induce a second neural axis in a host embryo after heterotopic transplantation (Kinder et al., 2001). Taken together, the descendants of the EGO, MGO and LGO are laid down along the A-P axis and secrete paracrine factors to pattern neighbouring tissues and instruct body axis formation in A-P, dorsal-ventral (D-V) and left-right (L-R) fashion.

Before gastrulation starts at E6.5, the SRY HMG-box transcription factor Sox17 is expressed in the primitive endoderm (PrE), exVE and embryonic visceral endoderm (embVE), including the AVE and PVE (Burtscher et al., 2012; Kanai-Azuma et al., 2002; Niakan et al., 2010). The cells fated to become DE emerge from a $Foxa2^+$ epiblast population (Burtscher and Lickert, 2009), ingress into the PS region and start to express *Sox17* while they intercalate into and disperse the overlying VE (Burtscher et al., 2012; Kwon et al., 2008). After gastrulation, the Sox17 protein is restricted to the DE, the mid- and hindgut endoderm and the endothelial cells of the forming vasculature (Burtscher et al., 2012; Engert et al., 2009; Kanai-Azuma et al., 2002). Deletion of the *Sox17* gene results in turning defects and posterior truncations at E8.5-9.5 due to defective mid- and hindgut development (Kanai-Azuma et al., 2002; Viotti et al., 2012).

The genetic deletion of β-catenin in epiblast progenitors leads to ectopic cardiac mesoderm at the expense of DE formation, indicating that Wnt/β-catenin signalling is essential for the specification of mesoderm and endoderm from still plastic epiblast progenitor cells (Lickert et al., 2002). β-catenin has a dual cellular function in mediating cell-cell adhesion and canonical Wnt signal transduction (Aberle et al., 1996; Clevers and Nusse, 2012; MacDonald et al., 2009; Tanaka et al., 2011). In the absence of a Wnt ligand, β-catenin is degraded via the ubiquitin-proteasome pathway (Aberle et al., 1997). If Wnt binds to the seven-transmembrane receptor Frizzled, the destruction complex is inhibited and β-catenin accumulates in the cytoplasm, translocates to the nucleus and activates target genes by providing the transactivation domain for the Lymphoid enhancer factor (Lef) and T-cell factor (Tcf) transcription factor family. *In vitro* and *in vivo* studies suggest an interaction of β-catenin and Sox17 to activate endoderm target genes in *Xenopus laevis* and colorectal cancer cell lines (Sinner et al., 2007; Sinner et al., 2004). In mouse, several of the Wnt target genes identified as activated by Tcf and β-catenin regulate mesendoderm induction and specification, such as *Cdx1* (Lickert et al., 2000), brachyury (*T*) (Arnold et al., 2000; Yamaguchi et al., 1999) and *Foxa2* (Sawada et al., 2005). The Wnt/β-catenin target genes *T* and *Foxa2* are expressed in posterior epiblast progenitors in a mutually exclusive manner, suggesting that the T^+ mesoderm and $Foxa2^+$ mesendoderm descendants are already specified before gastrulation commences at E6.5 (Burtscher and Lickert, 2009). The combined activity of Nodal/TGFβ and Wnt/β-catenin signalling induces mesendoderm and PS formation (Brennan et al., 2001; Huelsken et al., 2000; Liu et al., 1999). To date, the function of Wnt/β-catenin signalling in the AVE, PVE and DE is not known owing to the early embryonic lethality of *Wnt3* and β-catenin mutants (Huelsken et al., 2000; Lickert et al., 2002; Liu et al., 1999).

To address the function of Wnt/β-catenin signalling in endoderm formation and embryonic patterning, we used a conditional knockout (CKO) strategy to delete β-catenin specifically in the Sox17-positive VE, DE and vascular endothelial cells. CKO embryos are embryonic lethal due to head and tail truncations at E10.5. We found that initial vascularisation and foregut development are independent of β-catenin function, but mid- and hindgut formation is strongly impaired due to a failure of DE formation at E7.5-8.5. Interestingly, rescue of the VE defects by tetraploid complementation revealed that the AVE and PVE are important for head and tail organizer formation, respectively. We show that PVE cells are located directly above the PS during gastrulation and tail bud elongation. β-catenin deletion in the PVE, but not in the epiblast, leads to a failure of gastrula organizer gene induction in a non-cell-autonomous manner. Finally, we show that Wnt/β-catenin signalling via Tcf4 activates *Sox17* during endoderm formation in differentiating embryonic stem cells (ESCs) and is necessary for *Sox17* expression in the VE and DE. Taken together, these results indicate that Wnt/β-catenin signalling via Sox17 regulates endoderm and organizer formation in cell-autonomous and non-autonomous manners, respectively.

MATERIALS AND METHODS

Generation of mutant mice and genotyping

The $Sox17^{2AiCre}$ allele, the β-catenin (*Ctnnb1*) floxed (*F*) and floxed deleted (*FD*) alleles were previously described (Brault et al., 2001; Engert et al., 2009; Lickert et al., 2002). Heterozygous $Sox17^{2AiCre}$; $Ctnnb1^{FD}$ mice were mated to homozygous $Ctnnb1^{F}$; *R26R* animals (Soriano, 1999). Mutant mice were bred on a mixed CD1×C57Bl/6 background. PCR genotyping was performed on tail tip genomic DNA and embryonic tissue following lysis in buffer containing proteinase K as described (Engert et al., 2009).

ESC derivation

$Sox17^{2AiCre/+}$; $Ctnnb1^{FD/F}$; $R26^{R/+}$ conditional mutant and $Sox17^{2AiCre/+}$; $Ctnnb1^{F/+}$; $R26^{R/+}$ control ESCs were generated from embryos obtained from the crossing scheme described above according to standard protocols.

Generation of expression vectors

The cDNA encoding td-tomato fluorescent protein (Shaner et al., 2004) was amplified by PCR using the following primers (5′-3′; restriction sites underlined): Tomato fwd (5′-*Xba*I), TCTAGAATGGTGAGCAAGGGCGAGGAG; Tomato rev (5′-*Spe*I), ACTAGTTTACTTGTACAGCTCGTCCATGCCG. *Xba*I/*Spe*I-digested PCR product was cloned into the pBluescript KS vector (Stratagene). The gene encoding histone 2B (H2B) was amplified by PCR using the following primers: H2B fwd (5′-*Not*I-Kozak-ATG-H2B), GCGGCCGCGCCACCATGCCAGAGCCAGCG; H2B rev (5′-*Xba*I-H2B), TCTAGACTTAGCGCTGGTGTACTTGGTGATGG. The amplified sequence was digested with *Not*I/*Xba*I and subcloned in front of the td-tomato cDNA in the pBluescript KS vector. The *Not*I/*Spe*I-digested H2B-td-tomato fluorescent marker was subcloned into the *Not*I/*Nhe*I sites of the eukaryotic expression vector pCAGGS (Niwa et al., 1991).

Generation of fluorescent reporter cell lines

The fluorescent ESC lines were generated by electroporation of *Eco*RV-linearised pCAGGS vector containing H2B-td-tomato into β-catenin CKO and control ESCs. Cells were selected on puromycin-resistant mouse embryonic fibroblasts and screened for ubiquitous reporter expression *in vivo* using embryos derived from ESCs.

Generation of tetraploid chimeras

Tetraploid chimeras were generated according to standard protocols (Nagy et al., 1993). Embryos were collected from donor mice expressing YFP (Hadjantonakis et al., 2002).

β-galactosidase staining and histology

β-galactosidase staining was performed as described (Liao et al., 2009). The embryos were dehydrated through an ethanol series and embedded in paraffin. Blocks were sectioned at 4-8 μm, collected on glass slides, dewaxed and counterstained with Nuclear Fast Red. Documentation was carried out using a Zeiss Stereo Lumar.V12 microscope.

Immunofluorescence

Immunostaining on whole-mount embryos was performed as previously reported (Burtscher and Lickert, 2009). The following antibodies were used: Foxa2 (sc-6554, Santa Cruz Biotechnology) 1:1000; Foxa2 (ab40874, Abcam) 1:1000; Sox17 (GT15094, Acris/Novus) 1:500; brachyury (sc17743, Santa Cruz Biotechnology) 1:500; β-catenin (C2206, Sigma) 1:2000; β-catenin (610154, BD) 1:2000; active β-catenin (05-665, Millipore); E-cadherin (610181, BD) 1:2000; RFP (600-401-379, Biotrend) 1:1000; GFP (GFP-1020, Aves Labs) 1:1000; Pecam1 (553370, BD) 1:500; Otx2 (AB9566, Chemicon) 1:200; and phospho-histone H3 (06-570, Millipore) 1:500. Immunostainings were analysed with a Leica laser-scanning SP5 confocal microscope (20× and 63× objectives).

TUNEL assay

Apoptosis detection was carried out using the In Situ Cell Death Detection Kit, Fluorescein (11684795910, Roche).

Whole-mount *in situ* hybridisation

In situ hybridisation on whole-mount embryos was performed as previously described but excluding proteinase K digestion (Lickert and Kemler, 2002).

Endoderm differentiation

IDG3.2 ESCs (Hitz et al., 2007) were passaged twice on gelatin-coated dishes before differentiation. Endoderm differentiation efficiency was monitored by antibody staining against Foxa2 and Sox17. ESCs were harvested and washed once with PBS. 1.6×10^7 cells were seeded onto a gelatin-coated 15-cm dish in SFO3 medium (Yasunaga et al., 2005) containing 10 ng/ml activin A (338-AC, R&D Systems) and 1 ng/ml Wnt3a (1324-WN, R&D Systems). Cells were differentiated for 4 days with a daily change of medium.

Chromatin immunoprecipitation (ChIP) assays

Preparation and immunoprecipitation of chromatin were according to published methods with modifications (Kagey et al., 2010). For ChIP, 5 μg anti-β-catenin (9652, Cell Signaling) or anti-Tcf4 (2565, Cell Signaling) antibody was used. Quantitative PCR (qPCR) was performed with the Fast SYBR Green Master Mix qPCR Kit (Applied Biosystems) using 10 μl of total reaction, and analysed on a Light Cycler 480 Real-Time PCR System (Roche Applied Science). For qPCR primers see supplementary material Table S1.

RESULTS

Conditional knockout of β-catenin in the Sox17⁺ endoderm and vascular endothelial lineage

Wnt/β-catenin signalling has been implicated in PS formation, mesendoderm specification and organizer induction (Huelsken et al., 2000; Lickert et al., 2002; Liu et al., 1999); however, the function of Wnt/β-catenin signalling in the embryonic and extra-embryonic endoderm lineages remains ill defined. As revealed by whole-mount immunohistochemistry (IHC) using antibodies against Foxa2 and Sox17 and laser-scanning microscopy of fixed embryos, Sox17 is strongly expressed in the exVE, AVE and PVE at E6.5 (Fig. 1A,B; supplementary material Fig. S1) and in the nascent DE at E7.5-7.75 (Fig. 1C,D), consistent with previous findings (Burtscher et al., 2012; Kanai-Azuma et al., 2002). At E8.5, Sox17 protein is most abundant in the mid- and hindgut endoderm and is restricted to the forming vascular endothelial cells (Burtscher et al., 2012).

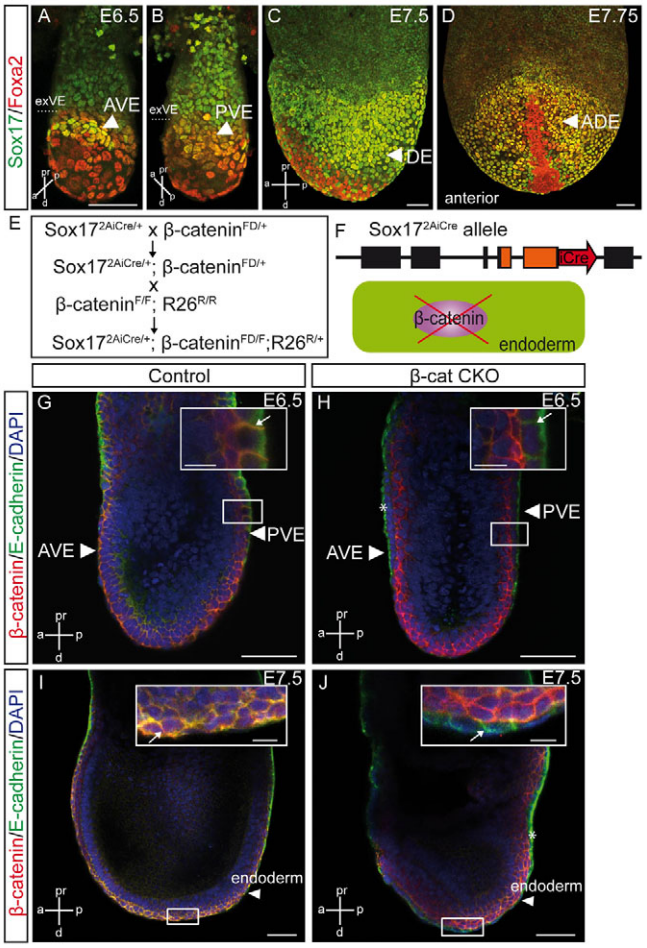

Fig. 1. Conditional knockout (CKO) of β-catenin in the Sox17 endoderm lineage. (**A-D**) Immunohistochemistry (IHC) using Sox17 and Foxa2 antibodies on wild-type (WT) mouse embryos at E6.5-7.75. Sox17 is localised to the extra-embryonic visceral endoderm (exVE), anterior visceral endoderm (AVE, arrowhead in A) and posterior visceral endoderm (PVE, arrowhead in B). Foxa2 is localised to the embryonic visceral endoderm (embVE), AVE and PVE at E6.5 (A,B). At E7.5 Sox17 is expressed in definitive endoderm (DE, arrowhead in C), while Foxa2 is also expressed in the anterior mesendoderm (AME) (C). Sox17 marks the anterior definitive endoderm (ADE, arrowhead in D), Foxa2 the ADE and AME at E7.75 (D). (**E**,**F**) Crossing scheme to generate β-catenin CKO embryos containing the *R26R* allele (E) using the *Sox17^{2AiCre}* allele (F). (**G-J**) Optical sections of antibody staining against β-catenin and E-cadherin at E6.5 (G,H) and E7.5 (I,J) confirm β-catenin deletion in the VE (H, arrowheads) and endoderm (J, arrowhead) of CKOs. Insets show higher magnification of boxed regions; arrows indicate presence (WT) or absence (CKO) of β-catenin. Asterisk indicates nonspecific E-cadherin staining. Scale bars: 50 μm, except 10 μm in insets.

To delete β-catenin in the Sox17 lineage, we used a crossing scheme in which males heterozygous for a β-catenin (*Ctnnb1*) floxed deleted (*FD*) allele (Brault et al., 2001; Lickert et al., 2002) and the *Sox17^{2AiCre}* allele (Engert et al., 2009) were mated to females homozygous for the β-catenin floxed (*F*) allele and the R26 reporter (*R26R*) allele (Fig. 1E,F) (Soriano, 1999). One-quarter of the offspring were CKOs, in which only one β-catenin floxed (*F*) allele had to be recombined by the Cre recombinase, while littermates

who inherited some but not all of the alleles served as controls (hereafter termed control and β-catenin CKO). We confirmed efficient gene deletion in CKO embryos in the VE at E5.5 and E6.5, as well as in the endoderm at E7.5 (Fig. 1G-J; supplementary material Fig. S2). Whereas β-catenin was localised to E-cadherin^{+} adherens junctions (AJs) in the VE and DE of control embryos, β-catenin deletion was uniform and restricted to both extra-embryonic and embryonic endoderm lineages in β-catenin CKO embryos at E5.5 to E7.5 (Fig. 1G-J, boxed area; supplementary material Fig. S2). Consistent with previous findings, epithelial integrity was maintained and E-cadherin was localised normally to the basolateral membrane compartment in the endoderm of β-catenin CKO embryos, indicating that plakoglobin can substitute for β-catenin to mediate cell-cell adhesion (Haegel et al., 1995; Huelsken et al., 2000; Lickert et al., 2002).

Lack of β-catenin affects endoderm and organizer formation

Next, we analysed embryos from gastrulation to somite stage by gross morphology, histology and Sox17 lineage tracing using the *R26R* allele. At E8.5, β-catenin CKO embryos showed clear truncations of the anterior head and posterior neural tube (NT) structures (n=16; Fig. 2A,B, red arrows). The lack of anterior neural induction can be explained by defects in the head organizer tissues, such as AVE, anterior mesendoderm (AME) and/or ADE. The posterior NT truncations were unexpected because we deleted β-catenin only in the endoderm and vascular endothelial cells as revealed by the *R26R* genetic lineage tracing at E8.5 (Fig. 2A,B). These results suggest that the posterior embryonic or extra-embryonic endoderm acts in a paracrine fashion to induce posterior neural structures (see below).

To follow the fate of the β-catenin mutant cells in more detail, we analysed embryos at earlier time points during gastrulation and early somite stage. As expected, Sox17 lineage cells were uniformly labelled in the embryonic and extra-embryonic endoderm of control embryos at E7.5-8.0 (Fig. 2C-E). By contrast, β-catenin CKOs showed an accumulation of AVE/ADE cells in the anterior region at E7.5 (n=7; Fig. 2F,H′), indicating that AVE and/or ADE formation is defective. During nascent DE formation we noticed an accumulation of Sox17 lineage mutant cells in the PS region at E7.75 (n=6; Fig. 2G) and a lack of Sox17 lineage cells in the lateral and posterior DE region at E8.0 (n=7; Fig. 2H), indicating that DE cells are initially specified but fail to contribute to the endoderm germ layer. This was further confirmed by marker analysis revealing some Sox17 and β-catenin double-positive cells in the ADE region at E7.75 (supplementary material Fig. S3). To investigate the fate of the Sox17 lineage DE cells, we analysed apoptosis and proliferation but did not detect any significant changes in the mutants at E7.5 (supplementary material Fig. S4). These results are consistent with previous results obtained with *Sox17* mutants (Kanai-Azuma et al., 2002) and implicate that Sox17 lineage cells are lost between E7.5 and E8.5.

Taken together, these results suggest that β-catenin is cell-autonomously required for AVE and DE formation and that non-cell-autonomous defects lead to head and tail truncations.

Lack of β-catenin affects VE patterning and DE formation

To confirm the defects in AVE formation and failure of anterior neural induction, we investigated the expression of specific marker genes. Sox17 was not detectable by IHC in the AVE region at E6.5 (n=4; Fig. 6B,D). We then analysed Otx2, which is normally

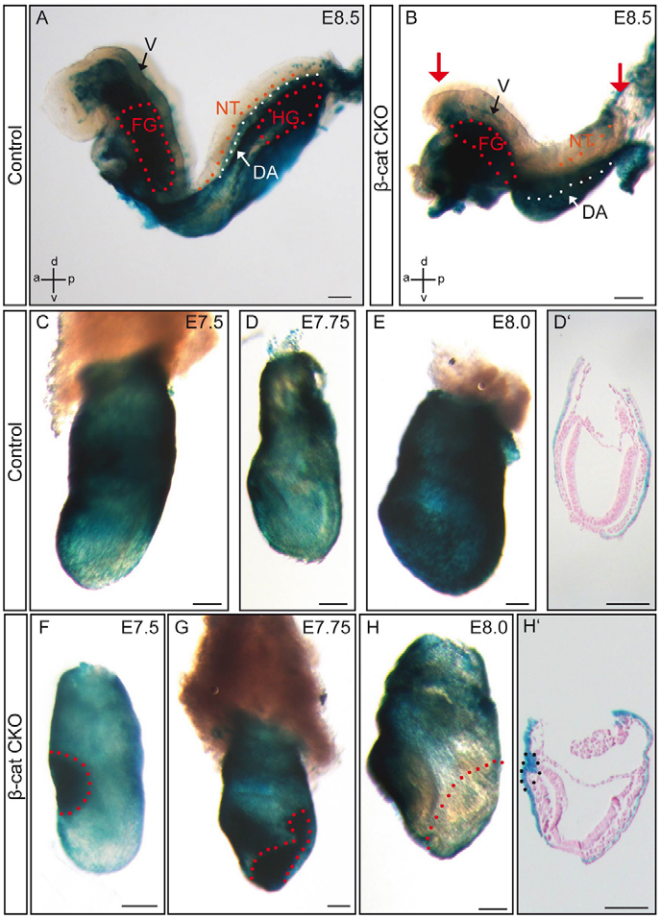

Fig. 2. Genetic lineage tracing reveals cell-autonomous and non-autonomous phenotypes in β-catenin CKO mouse embryos. (**A**) Sox17 lineage *lacZ*$^{+}$ cells contribute to the vascular system (V), including the dorsal aorta (DA, white dots), the foregut (FG), midgut and hindgut (HG) endoderm in controls at E8.5. (**B**) β-catenin CKO embryos show non-cell-autonomous head and neural tube (NT) truncations (red arrows) and cell-autonomous loss of hindgut structures. (**C-H′**) *lacZ* expression in control (C-E) and CKO (F-H) embryos from E7.5-8.0 demonstrate an accumulation of Sox17 lineage cells in the AVE/ADE at E7.5 (F,H′, dotted area), PS at E7.75 (G, dotted area) and absence of mutant cells in the posterior region at E8.0 (H, dotted area). (D′,H′) Paraffin sections of D and H. Anterior is to the left. Scale bars: 100 μm.

localised in the AVE and anterior epiblast and is important for head organizer formation. Otx2 is localised throughout the VE and strongly reduced in the epiblast region in CKO embryos at E7.0 (n=3/4; Fig. 3A,B), which was confirmed by whole-mount *in situ* hybridisation (WISH) at E7.5 (n=3; Fig. 3I,J). Moreover, analysis of the AVE marker genes *Cerl* and *Hex* (*Cer1* and *Hhex* – Mouse Genome Informatics) suggests AVE migration defects in β-catenin CKOs (n=2; supplementary material Fig. S5). We confirmed the failure of anterior neural induction by measuring the distance from the rostral forebrain to rhombomeres 3 and 5 of the caudal hindbrain as marked by *Krox20* (*Egr2* – Mouse Genome Informatics) ISH in control and β-catenin CKO embryos at E8.5 (n=5; Fig. 3K,L).

Next, we analysed the consequences of β-catenin deletion in the DE at E7.5-8.5. Previously, we described that loss of β-catenin in the epiblast progenitors of the AME and DE leads to ectopic cardiac mesoderm at the expense of DE formation (Lickert et al., 2002). To investigate a potential cell lineage switch in endoderm-specific β-

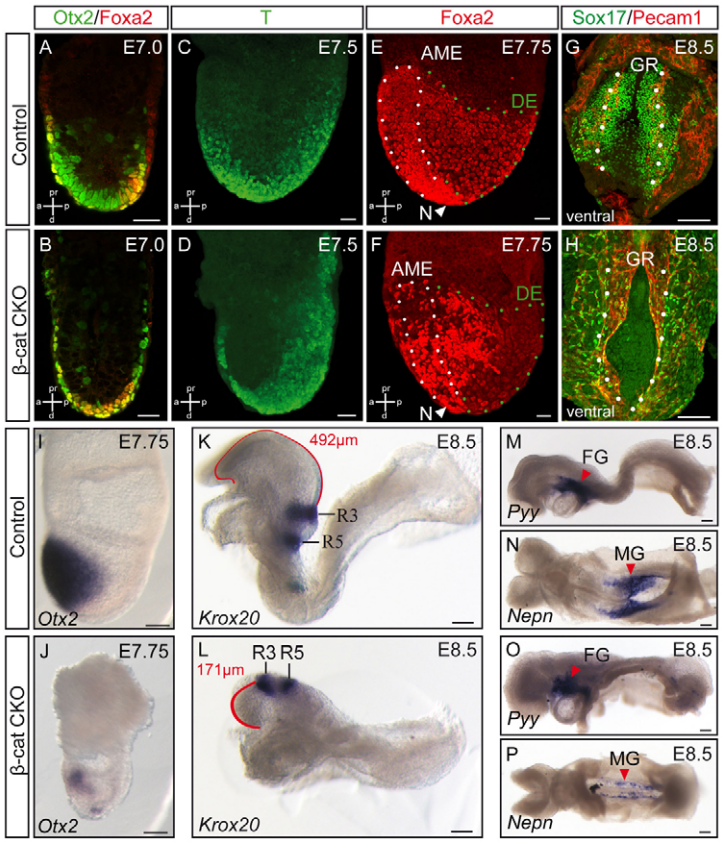

Fig. 3. Lack of β-catenin in the endoderm affects AVE and DE formation. (**A-H**) IHC for the indicated proteins demonstrates reduced Otx2 levels in the epiblast at E7.0 (A,B), normal T localisation in the AME and mesoderm (C,D), reduction of Foxa2 expression in the DE (green dotted area) at E7.5 (E,F; white dotted area indicates the AME, N the node) and absence of Sox17 in the gut region (GR, dotted area) at E8.5 (G,H) in β-catenin CKO mouse embryos. (**I-P**) Whole-mount *in situ* hybridisation (WISH) with the indicated probes at the indicated embryonic stages. *Otx2* mRNA is reduced in the anterior epiblast in CKOs (J) compared with controls (I) at E7.5. Lack of anterior structures is shown by the reduced distance of forebrain to hindbrain rhombomere R3 in the CKO (L) compared with control (K). CKO aggregation chimeras show (O) normal *Pyy* expression in the foregut (FG) and (P) reduced *Nepn* expression in the midgut (MG) compared with controls (M,N). Anterior is to the left. Scale bars: 25 μm in A-F; 100 μm in G-P.

catenin CKO embryos, we analysed the expression of the mesendoderm markers T and Foxa2 (Burtscher and Lickert, 2009). T marks posterior epiblast mesendoderm progenitors, AME and mesoderm, whereas Foxa2 marks anterior epiblast mesendoderm progenitors, AME and DE at E6.5-7.5. T^+ cells were formed in comparable numbers in the AME and mesoderm in control and β-catenin CKO embryos (n=9; Fig. 3C,D; supplementary material Fig. S5), whereas the number of $Foxa2^+$ cells in the AME, node, lateral and posterior DE was reduced in the CKO at E7.5 (n=20; Fig. 3E,F). Lack of DE formation resulted in the absence of $Sox17^+$ mid- and hindgut endoderm in β-catenin CKO embryos, while co-expression of Pecam1 and Sox17 in the vasculature suggested the normal development of vascular endothelial cells at E8.5 (n=2; Fig. 3G,H; supplementary material Fig. S6).

As indicated by the Sox17 genetic lineage tracing (Fig. 2), these results clearly show that β-catenin is essential for lateral and posterior DE formation. They further demonstrate that $Sox17^+$ cells are already restricted to the endoderm lineage, as no cell fate switch to cardiac mesoderm occurred (Lickert et al., 2002). However, the failure of Foxa2 induction in epiblast progenitors of the Sox17 lineage was unexpected (Engert et al., 2009) and suggests that both cell-autonomous and non-autonomous mechanisms contribute to the CKO mutant phenotype.

Tetraploid rescue reveals cell-autonomous and non-autonomous functions of β-catenin in organizer and DE formation

To analyse the cell-autonomous requirement of β-catenin in the extra-embryonic and embryonic endoderm, we used tetraploid (4n) embryo ↔ ESC aggregations to generate completely ESC-derived embryos (Tam and Rossant, 2003). In 4n embryo ↔ ESC aggregation chimera, the ESCs can only contribute to the embryonic epiblast, giving rise to the ectoderm, mesoderm and DE. By contrast, the extra-embryonic lineages, including VE and ExE, are formed by wild-type (WT) 4n cells of the host embryo. First, we generated several control and β-catenin conditional mutant ESC lines that were stably transfected with a ubiquitously expressed red fluorescent protein reporter transgene to trace the ESC contribution to chimeric embryos (see Materials and methods). Next, we aggregated these control and CKO ESCs with 4n WT ubiquitous yellow fluorescent protein (YFP)-expressing embryos. These experiments revealed that β-catenin is cell-autonomously required in the extra-embryonic AVE for head induction, as all 4n WT embryo ↔ CKO ESC aggregation chimera showed rescue of the anterior head truncation phenotype at E8.0-8.5 (n>30; compare Fig. 4A,F). Moreover, Sox17 genetic lineage tracing using the *R26R* allele of control and CKO ESCs indicated that β-catenin is neither essential for the formation of the dorsal aorta and vasculature (Fig. 4B,G; supplementary material Fig. S6) nor for the formation of the foregut in ESC-derived embryos (Fig. 4C,H). By contrast, β-catenin in the DE was crucially important for mid- (Fig. 4D,I) and hindgut (Fig. 4E,J) formation.

To analyse the degree of gut tube defects in extra-embryonic endoderm rescued chimera, we analysed the expression of *Pyy* and *Nepn* as marker genes for the fore- and midgut, respectively, by WISH at E8.5 (Fig. 3M-P) (McKnight et al., 2010). This revealed that foregut formation appeared normal (n=4; Fig. 3M,O) but that the lateral region of the midgut (n=6) (Fig. 3N,P) and hindgut (Fig. 4E,J) did not develop in the CKO mutant chimera at E8.5. Surprisingly, the posterior NT truncation phenotype was rescued in 4n WT embryo ↔ CKO ESC aggregation chimera (n>30), although the lateral mid- and hindgut endoderm tissue was almost completely missing (compare Fig. 4A,F, Fig. 2A,B and Fig. 3M-P), as quantified by measurement of the posterior tail region

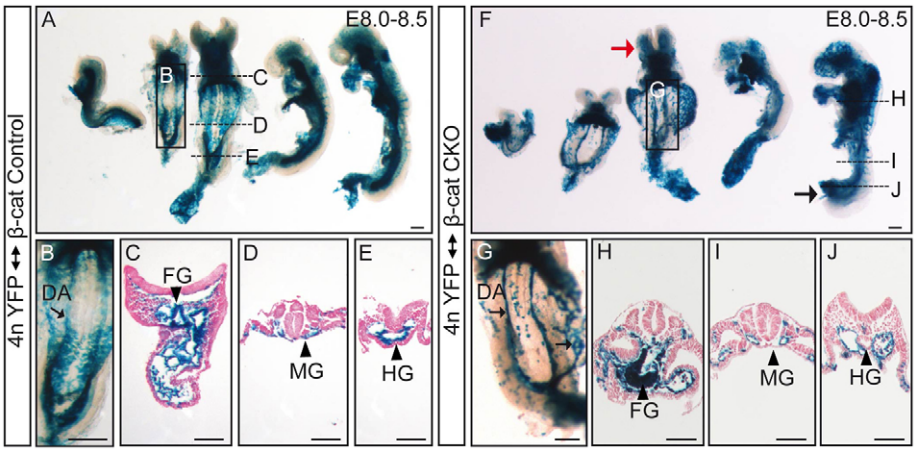

Fig. 4. Wild-type VE rescues head and NT truncation in aggregation chimeras. (**A**,**F**) *lacZ* staining of control (A) and β-catenin CKO (F) aggregation chimeras at E8.0-8.5 demonstrates rescue of the head (red arrow) and tail (black arrow) truncation phenotype by WT VE. (**B-E**,**G-J**) Vascular endothelial cells, dorsal aorta (DA, arrows) and foregut (FG) are formed in control (B,C) and CKO aggregation chimeras (G,H). Midgut (MG) and hindgut (HG) formation of CKO aggregation chimeras (I,J) is impaired compared with controls (D,E). (B,G) Higher magnification of boxed regions and (C-E,H-J) paraffin sections at the indicated levels of embryos in A and F. Scale bars: 250 μm in A,B,F,G; 100 μm in C-E,H-J.

(supplementary material Fig. S7). This indicates that the WT PVE rescues the tail truncation phenotype in otherwise midgut- and hindgut-depleted aggregation chimera and raises the intriguing possibility that PVE cells are essential for posterior organizer formation in a β-catenin-dependent manner.

To strengthen this observation, we tracked the relative contribution of 4n embryo-derived VE and ESC-derived DE cells in aggregation chimera at E7.5-8.5. At E7.5, both control and CKO chimera showed virtually coherent epithelial sheets of YFP$^+$ PVE cells above the underlying PS (Fig. 5E-H). By contrast, whereas the anterior and lateral VE (YFP$^+$ RFP$^-$) were dispersed by DE cells (YFP$^-$ RFP$^+$) in control chimera, in CKO chimera the VE cells still formed sheets, confirming that lateral and posterior DE cells are not formed (*n*=20; Fig. 5A-H; quantified in supplementary material Fig. S8A-G). Further evidence that the PVE cells rescue the axis elongation defect comes from the fact that these cells still form an epithelial sheet underlying the posterior PS region in both control and CKO chimera at E8.0-8.5 (Fig. 5I-P; supplementary material Fig. S8H-U). Whereas Sox17$^+$ DE cells (YFP$^-$ RFP$^+$) had replaced almost all VE cells (YFP$^+$ RFP$^-$) in the mid- and lateral hindgut region in control chimera, CKO chimera showed no sign of Sox17$^+$ DE formation, but showed epithelial rupturing and a high contribution of Sox17$^+$ VE cells (YFP$^+$ RFP$^-$) at E8.5 (*n*=6; Fig. 5J-L,N-P; supplementary material Fig. S8, Movie 1). Interestingly, DE and VE cells are positive for Sox17 in control chimera, whereas the few remaining RFP$^+$ DE cells in the mid- and hindgut region of the CKO chimera are Sox17 negative and only the 4n-derived WT VE cells express Sox17 (Fig. 5K,O). We confirmed these results in control and CKO embryos derived from natural matings at E8.5. Although Sox17 protein was detectable in the forming vasculature,

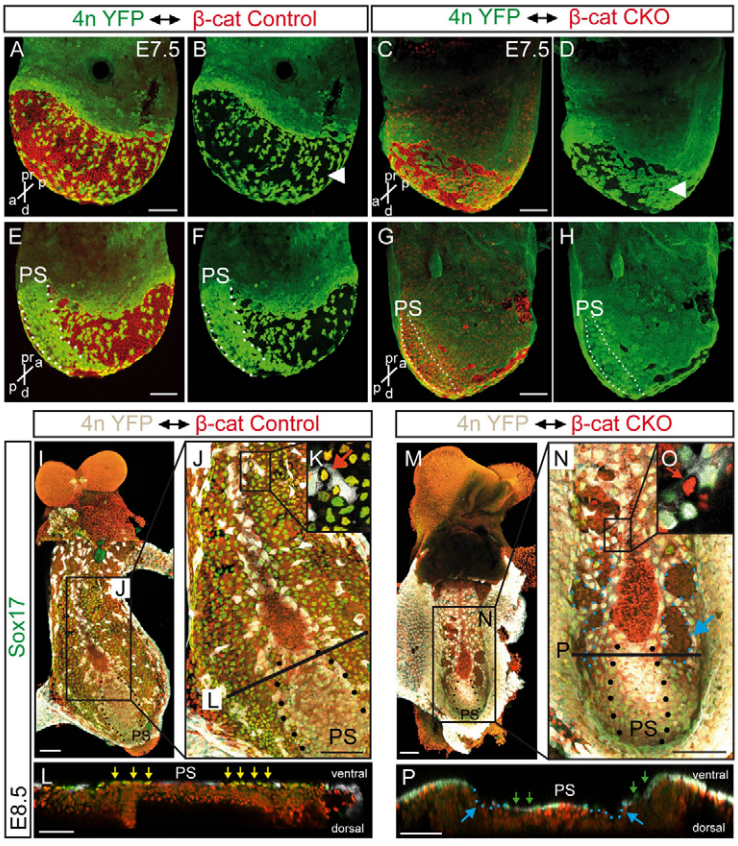

Fig. 5. RFP$^+$ β-catenin CKO cells rarely contribute to DE and midgut/hindgut formation. (**A-H**) IHC using GFP and RFP antibodies to distinguish between 4n YFP$^+$ host mouse embryo and RFP$^+$ ESC-derived cells, respectively. At E7.5, the YFP$^+$ VE in CKO aggregation chimeras (C,D, arrowhead) is not replaced by RFP$^+$ DE cells when compared with the control chimera (A,B, arrowhead). In both control (E,F) and CKO (G,H) chimeras, sheets of VE cells underlie the primitive streak region (PS, dotted area). (**I-P**) At E8.5, the VE cell layer is not replaced by DE (M-P) and shows rupturing in CKO chimeras (N,P, blue dotted area and arrows) compared with controls (J,L). VE cells underlie the PS (dotted area) in both control and CKO chimeras (I,J,M,N). VE cells in the gut of CKO chimeras express Sox17 (O,P, green arrows) in contrast to the few DE-like cells (compare O and K, red arrow), whereas in control chimeras the DE and remaining VE cells both express Sox17 (K,L, yellow arrows). Scale bars: 100 μm.

no expression was found in the mid- and hindgut (supplementary material Fig. S6A,B). This raises the possibility that *Sox17* might be a downstream target gene of the Wnt/β-catenin signalling cascade in the endoderm, but not in the vascular endothelial lineage. Taken together, these findings reveal that β-catenin is cell-autonomously required for AVE and DE formation and that the AVE and PVE display head and tail organizer function, respectively.

Wnt/β-catenin activates Sox17 via Tcf4 binding sites in the promoter and regulatory region

Consistent with the reported *Sox17* expression in PrE progenitors at E4.5 (Artus et al., 2011; Burtscher et al., 2012; Morris et al., 2010; Niakan et al., 2010), β-catenin deletion occurred throughout the VE, which coincided with the loss of Sox17 at E5.5-6.5 (n=5; Fig. 6A-D; supplementary material Fig. S2). At E7.25, a few cells synthesized Sox17 during DE formation (n=3; Fig. 6E-H), which was likely to be due to the delay in Cre-mediated recombination. Together, these results suggest that Wnt/β-catenin signalling is necessary for the maintenance of *Sox17* expression in the VE and DE.

To support this notion, we scanned the *Sox17* upstream and downstream regulatory regions for Tcf/Lef binding elements (TBEs) (Lickert et al., 2000) using Genomatix software. We found 13 TBEs in the 8945 kb upstream and downstream regulatory region, including intron 1 and 2 (Fig. 6I; supplementary material Table S2). The *Sox17* gene has several alternative transcription start sites that are used in a vascular endothelial and endoderm tissue-specific manner (Burtscher et al., 2012; Engert et al., 2009; Liao et al., 2009). To test directly whether these TBEs are occupied by Tcf4/β-catenin transactivation complexes, we used ChIP with antibodies to Tcf4 (Tcf7l2 – Mouse Genome Informatics) and β-catenin and analysed the enrichment on putative TBEs by qPCR. We used ESCs under pluripotency and endoderm differentiation conditions (Burtscher et al., 2012; Yasunaga et al., 2005). Strikingly, both Tcf4 and β-catenin could only be ChIPed in ESCs that had been induced by Wnt3a and activin to differentiate into endoderm, but were not bound to the TBEs under pluripotency conditions (Fig. 6J). These results strongly suggest that canonical Wnt signalling regulates *Sox17* via Tcf4/β-catenin complexes during endoderm formation (Fig. 6K).

PVE induces gastrula organizer genes in the epiblast in a Wnt/β-catenin-dependent manner

Wnt3 expression and Wnt/β-catenin activity are restricted to the posterior epiblast and PVE at E5.5-6.5 (Ferrer-Vaquer et al., 2010; Rivera-Pérez and Magnuson, 2005) and initiate A-P asymmetries and posterior mesendoderm formation (Chazaud and Rossant, 2006; Huelsken et al., 2000; Liu et al., 1999; Tortelote et al., 2013). Our analysis suggests that Wnt/β-catenin signalling via Sox17 is essential for VE patterning and posterior axis formation. To lend further support to this idea, we investigated the expression of the Wnt/β-catenin target gene *Foxa2* (Sawada et al., 2005). Foxa2

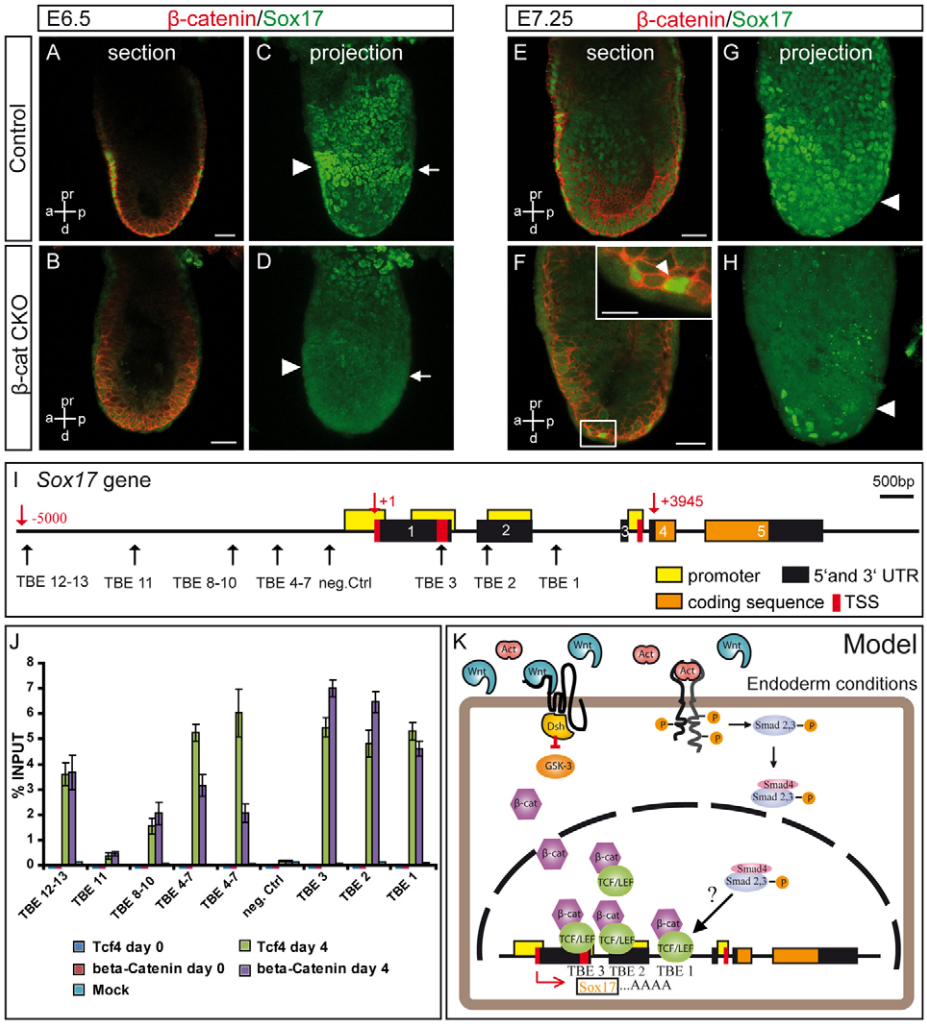

Fig. 6. *Sox17* is a downstream target gene of Wnt/β-catenin signalling in the endoderm. (**A-H**) Absence of Sox17 in the AVE (arrowhead) and PVE (arrow) at E6.5 (B,D) and in the DE (arrowhead) at E7.25 (F,H) in CKO versus control (A,C,E,G) mice. Inset in F shows higher magnification of Sox17/β-catenin-positive cells (arrowhead). Anterior is to the left. (**I**) The mouse *Sox17* gene showing Tcf/Lef binding elements (TBEs, black arrows) within the regulatory region from −5000 to +3945 bp (red arrow). Exons are numbered (1-5). TSS, transcription start site. (**J**) ChIP with antibodies against β-catenin and Tcf4 on ESCs under pluripotency conditions (d0) or endoderm differentiation conditions (d4) reveals strong accumulation of Tcf4 and β-catenin on TBE-containing fragments upon endoderm differentiation. Error bars indicate s.d. of four replicates. (**K**) Model: canonical Wnt signalling regulates *Sox17* via Tcf4/β-catenin complexes during endoderm formation. Upon binding of the Wnt ligand to its receptor the degradation complex is inhibited, β-catenin accumulates in the cytoplasm, translocates to the nucleus and together with Tcf4 activates the expression of *Sox17* in the endoderm. Scale bars: 25 μm, except 10 μm in inset.

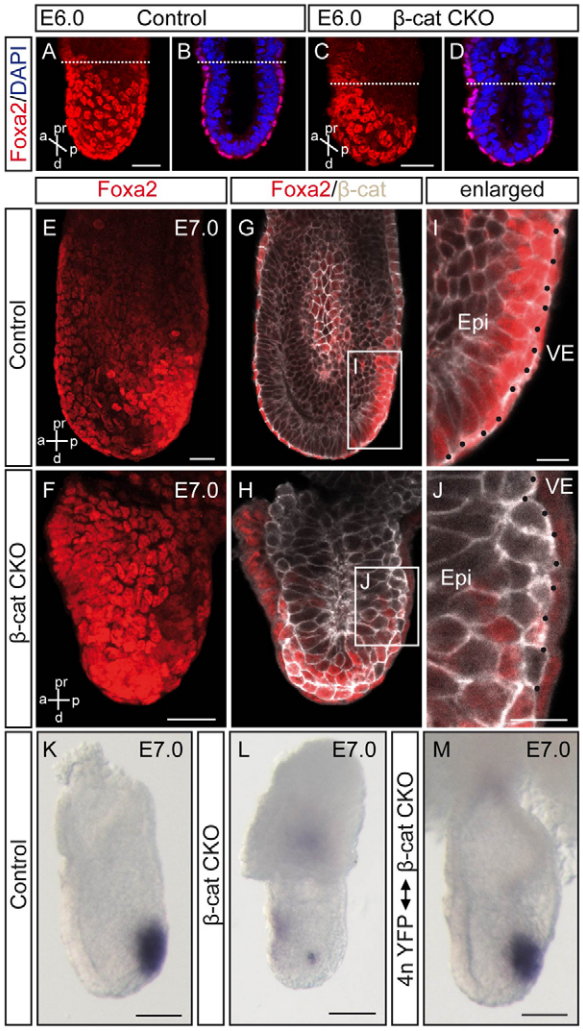

Fig. 7. PVE induces organizer gene expression in the posterior epiblast. (**A-D**) IHC reveals reduced levels of Foxa2 synthesis in the PVE of β-catenin CKOs at E6.0 (C,D) compared with controls (A,B). Dotted line indicates embryonic–extra-embryonic boundary). (**E-M**) IHC (E-J) and WISH (K-M) show reduced Foxa2 expression in the posterior epiblast in CKO (F,H,J,L) compared with control embryos (E,G,I,K), which is restored in CKO chimeras (M). (A,C,E,F) *z*-projections; (B,D,G,H) optical sections; (I,J) higher magnifications of the indicated boxed regions. Anterior is to the left. Scale bars: 25 μm in A-H; 10 μm in I,J; 100 μm in K-M.

protein was specifically absent in the PVE region at E6.0, suggesting that PVE identity was lost (Fig. 7A-D). We examined whether lack of PVE identity could influence posterior epiblast pattering in a non-cell-autonomous fashion. At E7.0, *Foxa2* mRNA and protein were not induced to normal levels in the proximal epiblast in CKO embryos (n=8), but this was completely rescued by WT VE in CKO chimeras (Fig. 7E-M). Consistently, expression of the gastrula organizer gene *Nog* was reduced in the node region of mutant embryos (supplementary material Fig. S5G-J). Taken together, these findings strongly suggest that paracrine signals from the PVE induce organizer gene induction in the posterior epiblast in a non-cell-autonomous fashion at E6.5-7.5.

DISCUSSION

In this study we analysed the function of β-catenin in the Sox17 lineage, namely the AVE, PVE, DE and vascular endothelial cells. We showed that AVE and PVE defects lead to failure in head and tail organizer formation in β-catenin CKOs. Moreover, β-catenin is required for Sox17-dependent lateral and posterior DE formation and loss of β-catenin in the Sox17 lineage causes a mid- and hindgut phenotype. Interestingly, we showed that the activation and/or maintenance of *Sox17* expression in the endoderm depends on Wnt/β-catenin signalling in the embryo as well as in ESC endoderm differentiation in culture. Vascular endothelial cell and dorsal aorta formation appeared unaffected in conditional β-catenin mutants, indicating that β-catenin signalling and its cell adhesion function are dispensable for initial vessel formation. In vascular endothelial cells Sox17 was synthesized normally in β-catenin CKOs, an indication that other signalling pathways, e.g. Notch/RBPjκ signalling, independently regulate *Sox17* gene expression in this cell type. Thus, regulation of *Sox17* expression via the Wnt/β-catenin signalling pathway might account for all phenotypes observed in the β-catenin CKOs.

Although β-catenin has a dual cellular function in cell-cell adhesion and canonical Wnt signalling, we strongly believe that the CKO mutant phenotype is caused by impaired Wnt/β-catenin signalling for several reasons. First, E-cadherin is localised normally to the basolateral membrane of endoderm cells in CKO embryos at E5.5 to E7.5, indicating that AJs are formed normally. Furthermore, cell-cell adhesion is not only maintained in the AVE and PVE, but also vascular endothelial cells seem to form normal tubular structures. This indicates that cell-cell adhesion can be established and maintained without the adhesion function of β-catenin. Second, the epithelial integrity of the endoderm is maintained up to E8.5, when lack of organizer induction has already led to head and tail defects and the deficit in DE induction and formation has caused mid- and hindgut defects. At E8.5, the VE in the mid- and hindgut region shows rupturing, which is likely to be due to the failure to generate the appropriate number of DE cells in the epithelial layer of the mid- and hindgut. Third, we identified *Sox17* as a downstream target of Wnt/β-catenin signalling in endoderm differentiated from pluripotent ESCs, suggesting direct or indirect regulation in the AVE, PVE and DE of the mouse embryo. Loss of Sox17 expression in the AVE, PVE and DE occurs in regions of Wnt signalling and in the absence of any obvious epithelial defects. As target gene activation and/or maintenance is already affected before a morphological phenotype is visible, this strongly suggests that the CKO phenotype is caused through loss of β-catenin function in canonical Wnt signalling. Finally, previous *in vivo* studies have provided evidence that plakoglobin can substitute for β-catenin to mediate cell-cell adhesion (Haegel et al., 1995; Huelsken et al., 2000; Lickert et al., 2002).

Wnt/β-catenin signalling is essential for head and tail organizer formation

Asymmetric localised Lefty1-expressing PrE cells give rise to the DVE at E5.5 (Takaoka et al., 2011); however, it is currently unclear which signals regulate the translocation of the PrE progenitors to the future DVE region. Subsequent to DVE induction, Wnt morphogen gradients are implicated in the movement of these cells to the anterior side of the embryo to form the AVE (Kimura-Yoshida et al., 2005). Although β-catenin is deleted in the entire VE by E5.5, we do not observe a lack of DVE marker gene induction, consistent with previous findings in β-catenin knockout (KO) embryos (Huelsken et al., 2000). Moreover, loss of β-catenin in the VE results in defects of AVE migration. Thus, our study suggests that tightly balanced levels of Wnt activity are important for VE pattering and AVE migration but are not required for initial DVE formation.

Several KO studies of AVE-specific genes and tissue ablation have demonstrated its importance for neural induction and maintenance of anterior epiblast identity (Beddington and Robertson, 1998; Perea-Gomez et al., 2001; Thomas and Beddington, 1996). Deletion of AVE-specific genes, such as *Hex* and *Dkk1* (Martinez-Barbera et al., 2000; Mukhopadhyay et al., 2001), affects anterior but not posterior axis development. However, in conditional β-catenin mutants we observe anterior and posterior axis truncations, implying that PVE is important for posterior axis formation. Evidence for this idea comes from several studies, including our own. First, Wnt3 is expressed in the PVE and lack of Wnt3 and β-catenin leads to failure of A-P axis formation (Huelsken et al., 2000; Liu et al., 1999). Additionally, Wnt signalling has been shown to be active in the PVE region at the time of P-D axis conversion at E5.5-6.5 (Ferrer-Vaquer et al., 2010). We show that the Wnt/β-catenin downstream target gene *Foxa2* is specifically downregulated in the PVE and Sox17 fails to be maintained throughout the VE in β-catenin CKOs. Finally, lack of PVE identity leads to a non-cell-autonomous failure of organizer gene induction in the epiblast that can be rescued by WT VE. Taken together, these results indicate that the PVE secretes paracrine factors that induce gastrula organizer genes in the adjacent epiblast, analogous to the Nieuwkoop centre in *Xenopus* and the posterior marginal zone in chick embryos (Bachvarova et al., 1998; Harland and Gerhart, 1997; Nieuwkoop, 1969). Interestingly, comparative fate maps of amphibian and chick embryos place the analogous structure of the Nieuwkoop centre in the posterior proximal region of the mouse embryo at E5.5-6.5 (Beddington and Robertson, 1999; Tam and Behringer, 1997).

The nascent DE at E7.0-7.5 and the mid- and hindgut seem to have little effect on posterior axis formation, as the tail truncation phenotype can be rescued by WT PVE in the absence of mid- and hindgut formation. This can be explained by the fact that DE cells form at E7.0 in the anterior PS region, intercalate at a distal position into the overlying VE and migrate to lateral and anterior regions to disperse the VE epithelial layer (Burtscher et al., 2012; Burtscher and Lickert, 2009; Viotti et al., 2012). By contrast, the PVE remains a virtually coherent epithelial sheet overlying the PS from E6.5-8.5. As such, the PVE is perfectly positioned and in direct contact with the posterior medial epiblast to secrete paracrine factors that induce and maintain organizer gene expression. We speculate that these secreted factors are downstream of the Wnt/β-catenin signalling cascade and the endoderm-specific transcription factor Sox17, as both β-catenin CKO and *Sox17* KO embryos experience posterior truncations that can be specifically rescued by WT PVE in the β-catenin mutants. We also suggest that the Nieuwkoop centre analogue PVE is induced by Wnt/β-catenin signalling, comparable to in the amphibian embryo. The secreted morphogen Wnt3 would be a likely candidate, as outlined above and as previously suggested (Rivera-Pérez and Magnuson, 2005).

Wnt/β-catenin signalling via Sox17 is required for DE formation

In addition to the important function of Wnt/β-catenin signalling in VE patterning and organizer gene induction, our results also implicate canonical Wnt signalling in DE formation. Previously, we have shown by genetic lineage tracing that Foxa2 epiblast progenitors give rise to axial, cranial and cardiac mesoderm as well as to DE (Horn et al., 2012; Uetzmann et al., 2008). Wnt/β-catenin signalling is important to induce and specify mesendoderm epiblast progenitors to become DE (Huelsken et al., 2000; Lickert et al., 2002). Failure of Wnt/β-catenin-mediated gene induction in the epiblast progenitors leads to ectopic cardiac mesoderm formation at the expense of DE formation (Lickert et al., 2002). The endodermal transcription factor Sox17 is weakly expressed in DE progenitor cells that have already delaminated from the epiblast and is strongly upregulated when these cells intercalate into the outside VE (Burtscher et al., 2012). Our CKO analysis revealed that these $Sox17^+$ DE cells are already specified for the DE lineage, as no fate switch to the cardiac lineage occurred, in contrast to our previous observations (Lickert et al., 2002). The data also suggest that Wnt/β-catenin signalling regulates *Sox17* expression and an endoderm programme. Analysis of the *Sox17* upstream and downstream regulatory region revealed many TBEs, and Wnt/β-catenin-induced endoderm formation in ESC culture leads to specific accumulation of Tcf4 and β-catenin on these cis-regulatory elements. This demonstrates that, in the presence of Wnt ligands, β-catenin and Tcf4 translocate to the nucleus to activate the *Sox17* gene. Sox17 further activates an endoderm programme for the formation of the DE. Importantly, the β-catenin CKO and *Sox17* KO phenotypes are similar in terms of mid- and hindgut defects, which strongly suggests that all Wnt activity is mediated by activation of *Sox17* (Kanai-Azuma et al., 2002; Viotti et al., 2012). This also shows that the Cre-mediated recombination is very efficient, as only very few, if any, $Sox17^+$ cells can escape genetic deletion of β-catenin. It is interesting that the β-catenin CKO and *Sox17* KO still form ADE, which suggests that Wnt/β-catenin signalling via Sox17 activation is essential for lateral and posterior endoderm formation.

In summary, our results suggest that Wnt/β-catenin signalling is essential for head and tail organizer formation and is required for lateral and posterior DE formation. These findings are not only important to understand embryonic axis formation in the mouse, but are also highly relevant to the differentiation of ESCs towards appropriate endoderm lineages for cell-replacement therapies.

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Competing interests statement

The authors declare no competing financial interests.

Author contributions

S.E. performed the experiments and quantifications except for Fig.6J, which was done by S.D and G.S. W.P.L. bred the mouse lines and introduced S.E. to the project. I.B. generated the expression vectors. H.L. and S.E wrote the manuscript. H.L. directed the study.

Supplementary material

Supplementary material available online at
http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.088765/-/DC1

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