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Wnt Secretion from Epithelial Cells and Subepithelial Myofibroblasts Is Not Required in the Mouse Intestinal Stem Cell Niche In Vivo

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SUMMARY

Wnt signaling is a crucial aspect of the intestinal stem cell niche required for crypt cell proliferation and differentiation. Paneth cells or subepithelial myofibroblasts are leading candidate sources of the required Wnt ligands, but this has not been tested in vivo. To abolish Wnt-ligand secretion, we used *Porcupine* (*Porcn*) conditional-null mice crossed to strains expressing inducible Cre recombinase in the epithelium, including Paneth cells (*Villin-Cre^{ERT2}*); in smooth muscle, including subepithelial myofibroblasts (*Myh11-Cre^{ERT2}*); and simultaneously in both compartments. Elimination of Wnt secretion from any of these compartments did not disrupt tissue morphology, cell proliferation, differentiation, or Wnt pathway activity. Thus, Wnt-ligand secretion from these cell populations is dispensable for intestinal homeostasis, revealing that a minor cell type or significant and unexpected redundancy is responsible for physiologic Wnt signaling in vivo.

INTRODUCTION

Intestinal crypts house self-renewing stem cells and transit-amplifying progenitors that depend on Wnt signaling. Expression of endogenous pathway antagonists such as *Dickkopf-1* reduces Wnt signaling, arrests stem and progenitor cell proliferation, and impairs secretory cell differentiation (Kuhnert et al., 2004; Pinto et al., 2003). Additionally, although it is unclear exactly when Wnt signaling begins in the developing intestine (Kim et al., 2007), mice lacking *Tcf4*, a transcriptional effector of the Wnt pathway, show marked epithelial defects (Korinek et al., 1998; van Es et al., 2012). Conversely, constitutive Wnt activity drives excessive cell replication and tumors, including human colorectal cancer (Korinek et al., 1997; Morin et al., 1997). Intestinal stem cells (ISCs) located at the base of mouse small-intestine crypts express the Wnt-responsive gene *Lgr5* (Barker et al., 2007), which encodes a coreceptor for Wnt-agonist R-spondins (Carmon et al., 2011; de Lau et al., 2011; Glinka et al., 2011) and are the origin for epithelial tumors spurred by constitutive Wnt activity (Barker et al., 2009). Although Wnt signaling is thus an imperative aspect of intestinal homeostasis, the bona fide physiologic Wnt source within the ISC niche in vivo is unknown.

Current hypotheses for this source draw on diverse observations. Paneth cells are a favored epithelial source because they reside in intimate contact with Lgr5^{+} ISCs (Barker et al., 2007; Cheng and Leblond, 1974) and their coculture with the latter improves intestinal organoid formation (Sato et al., 2011). *Wnt3*, *Wnt6*, and *Wnt9b* are highly expressed at the crypt base, and *Wnt3*, in particular, is present at higher levels in Paneth cells than in ISCs (Gregorieff et al., 2005; Sato et al., 2011). However, partial (Bastide et al., 2007; Garabedian et al., 1997; Mori-Akiyama et al., 2007; Shroyer et al., 2005) or complete, irreversible (Durand et al., 2012; Kim et al., 2012) Paneth cell ablation preserves mouse-crypt homeostasis in vivo, suggesting extraepithelial Wnt sources. A likely mesenchymal source is the subepithelial myofibroblasts (SEMFs) that envelop intestinal crypts (Powell et al., 2011) and support intestinal epithelial growth ex vivo and in tissue xenografts, likely through Wnt secretion (Lahar et al., 2011). SEMFs and other subepithelial cells express several *Wnts*, including *Wnt2b*, *Wnt4*, and *Wnt5a* (Gregorieff et al., 2005). Non-SEMF mesenchymal cells, such as nonmuscle fibroblasts, endothelial cells, neurons, or blood leukocytes, could also provide the required Wnts. Finally, epithelial and subepithelial cells may serve as redundant sources, much as renal development and function reveal complex, overlapping sites of Wnt production (Kispert et al., 1998; Park et al., 2007).

We used *Porcupine (Porcn)* conditional knockout mice to identify the source of intestinal Wnts. *Porcn* encodes an O-acyltransferase that palmitoylates all vertebrate Wnts at a conserved serine residue and is necessary for their

secretion (Chen et al., 2009; Najdi et al., 2012; Proffitt and Virshup, 2012). *PORCN* mutations cause focal dermal hypoplasia in humans (Grzeschik et al., 2007; Wang et al., 2007), and the tissue defects in *Porcn*-null mice resemble those observed upon loss of single *Wnts*, including *Wnt3*, *Wnt3a*, *Wnt5a*, and *Wnt7b* (Barrott et al., 2011; Biechele et al., 2011, 2013; Liu et al., 2012). Thus, our approach circumvented the problem of redundancy that afflicts studies of single-*Wnt* gene disruption. We used *Villin-Cre^{ERT2}* mice (el Marjou et al., 2004) to delete *Porcn* in the intestinal epithelium and *Myh11-Cre^{ERT2}* mice (Wirth et al., 2008) for deletion in subepithelial cells. Loss of *Porcn* from all epithelial or smooth-muscle (including SEMF) cells, alone or in combination, produced none of the defects in crypt cell proliferation, differentiation, or Wnt target-gene expression expected from Wnt deficiency. This rigorous genetic study therefore points to some other minor cell type as a source of Wnt ligands in the mammalian intestine.

RESULTS AND DISCUSSION

To test if the Wnt ligand(s) required for intestinal homeostasis originate in epithelial (including Paneth) cells, we crossed *Villin-Cre^{ERT2}* and *Porcn$^{Fl/Y}$* mice (Figure 1A; Barrott et al., 2011; el Marjou et al., 2004). LoxP sites flank exons 2 and 3 in the *Porcn^{Fl}* allele, poised for Cre recombinase-mediated deletion of the first three transmembrane domains to produce a null allele. As *Porcn* is X-linked, hemizygote males carry a single null allele. Although *Villin-Cre* is active in Paneth cells (Kim et al., 2012), we examined *Porcn$^{Fl/Y}$;Villin-Cre^{ERT2}* (*Porcn$^{E\text{-}Del}$* for epithelium-deleted) males at least 8 weeks after tamoxifen (TAM) exposure to ensure that Paneth cells were derived from *Porcn*-null ISCs. PCR on genomic DNA and quantitative RT-PCR (qRT-PCR) on mRNA isolated from the small intestine epithelium verified efficient *Porcn* deletion (Figure 1B) and loss of *Porcn* transcripts (Figure 1C; note logarithmic scale). *Porcn$^{E\text{-}Del}$* mice thrived without weight loss or morbidity (Figure S1A available online), and tissue morphology and crypt cell replication were intact. Both Paneth cells and goblet cells, which require Wnt signaling to differentiate (Pinto et al., 2003; van Es et al., 2005), were undisturbed (Figures 1D, S1B, and S1C). Nuclear β-catenin was present in crypt base cells and the levels of well-characterized Wnt target mRNAs, including *Axin2*, *Myc*, *Cyclin D1*, *Cd44*, *Sox9*, and *Lgr5*, were equal in *Porcn^{E-Del}* and wild-type small-intestine crypt epithelium (Figure 1E; Table S1). These data reveal that intestinal epithelium is not an essential source of Wnt ligands in vivo.

To determine if subepithelial cells, such as SEMFs, are a required physiologic Wnt source, we used *Myh11-Cre^{ERT2}* mice. *Myh11*-encoded smooth-muscle myosin heavy chain is expressed in much of the intestinal subepithelium (Wirth et al., 2008). To define the precise expression domain, we crossed *Myh11-Cre^{ERT2}* to *Rosa26-lox-STOP-lox-YFP* (*Rosa26R*) reporter mice and treated with TAM. Yellow fluorescent protein (YFP) expression was evident in circular and longitudinal muscle layers as well as in SEMF 5 days post-Cre induction, colocalizing with α-smooth muscle actin (Figures 2A and S2). Most subepithelial cells expressed YFP, but the lamina propria was not totally marked, indicating that *Myh11-Cre^{ERT2}* is expressed in most, but not all, subepithelial lineages. We then crossed *Myh11-Cre^{ERT2}* and *Porcn$^{Fl/Y}$* mice to ablate Wnt-ligand secretion specifically in SEMF and smooth muscle (Figure 2B), while preserving *Porcn* expression in the epithelium (Figure 2C). To estimate the mesenchymal cell fraction with recombined *Porcn*, we genotyped *Porcn* alleles in unfractionated *Porcn$^{Fl/Y}$;Myh11-Cre^{ERT2}* (*Porcn$^{M\text{-}Del}$*) intestines, expecting no recombination in epithelial, serosal, endothelial, or blood cells, which lack *Myh11* expression. In this light, a substantial contribution from recombined null *Porcn* DNA implied efficient recombination in most *Myh11*+ cells (Figure 2D), a point we demonstrate with greater confidence below (Figure 3B). Three weeks following Cre activation in *Porcn$^{M\text{-}Del}$* males, we did not observe weight loss (Figure S1A), implying preserved intestinal function. Gross and microscopic intestinal anatomy were intact, and *Porcn$^{M\text{-}Del}$* did not differ from control mice in the numbers of proliferating Ki67+ crypt cells; Paneth or other secretory cells (Figures 2E, S1B, and S1C); expression of nuclear β-catenin; or levels of Wnt target genes in crypt epithelium (Figure 2F). Thus, disruption of a subepithelial *Myh11*+ cell source of Wnts also did not perturb intestinal homeostasis.

Intestinal organoid cultures from isolated Lgr5^{+} ISCs require supplemental WNT3A, which is thought to derive in vivo from Paneth cells (Sato et al., 2011). However, mouse intestines devoid of epithelial *Wnt3* are normal and coculture of Lgr5^{+} ISCs with mesenchymal cells can substitute for WNT3A in organoid formation, suggesting redundant Wnt sources (Farin et al., 2012). To test this idea in vivo, we crossed *Porcn$^{Fl/Y}$* mice onto a compound *Villin-Cre^{ERT2};Myh11-Cre^{ERT2}* background and treated conditional null mice with TAM to force *Porcn* loss from both compartments (*Porcn$^{EM\text{-}Del}$*). Three weeks post-Cre activation, we observed nearly complete recombination of the floxed mutant allele in unfractionated intestines (Figure 3A), producing total absence of *Porcn* mRNA in the epithelium (Figure 3B). Unfractionated intestines showed 40% less *Porcn* mRNA in *Porcn$^{M\text{-}Del}$* mice and 85% less in compound *Porcn$^{EM\text{-}Del}$* mice (Figure 3B). Together, these data reveal highly efficient *Porcn* depletion in both compartments, with the expected residual contribution from

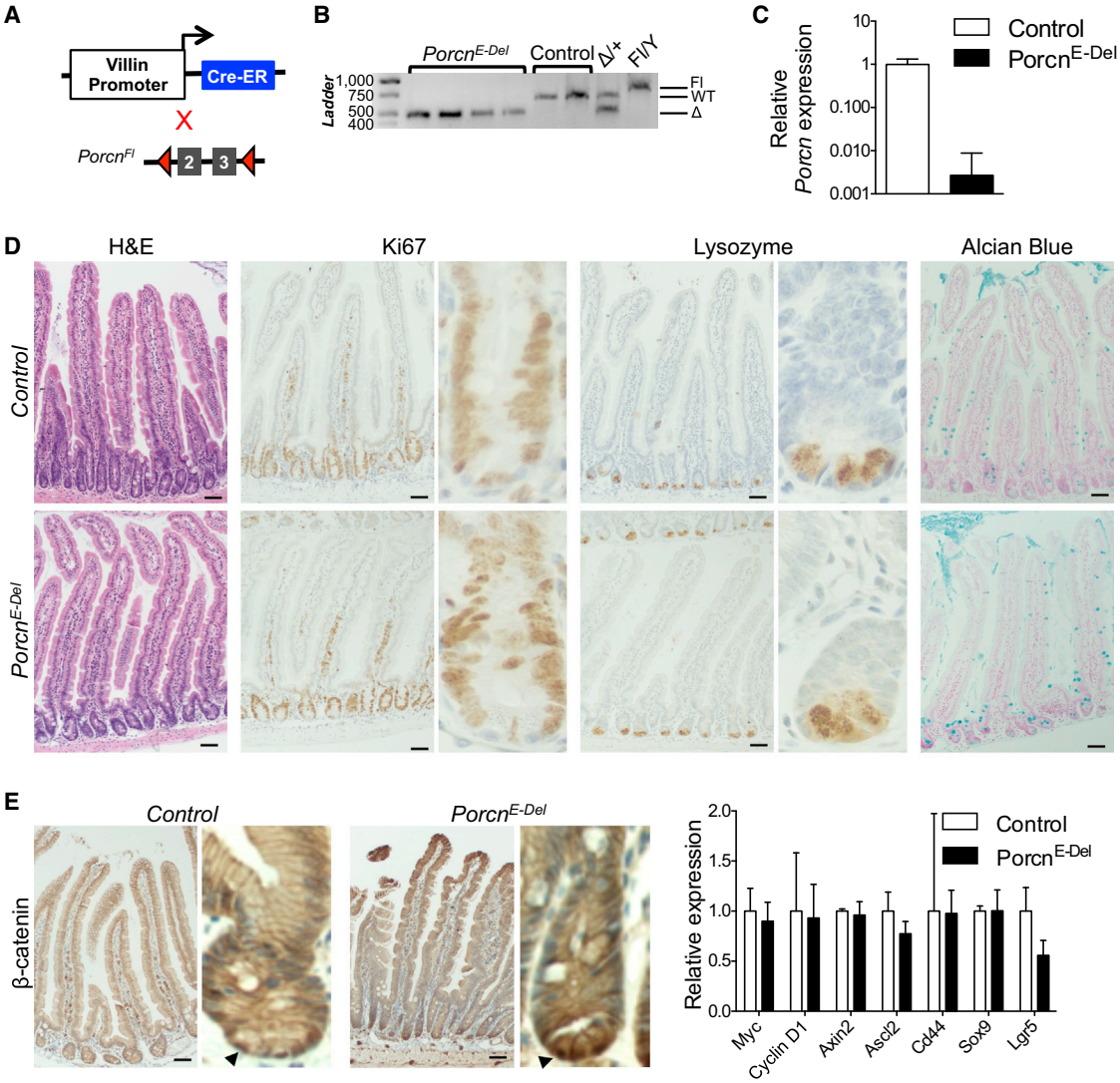

Figure 1. *Porcn* Deletion in Epithelial Cells Spares Intestinal Epithelial Morphology and Function

(A) Strategy for *Porcn* deletion by TAM-inducible Cre recombinase driven by epithelium-specific *Villin* promoter. ER, estrogen receptor.

(B) Genomic PCR of epithelium isolated from four $Porcn^{E\text{-}Del}$ intestines indicates complete recombination of the floxed allele (485 bp band) compared to two $Porcn^{+/Y}$ control mice (685 bp band from the wild-type allele). For reference, DNA is also shown from single $Porcn^{Del/X}$ (Δ/+; 485 bp and 685 bp products) and $Porcn^{Fl/Y}$;*Cre*− (762 bp band from the unrecombined floxed allele) mice. WT, wild-type.

(C) qRT-PCR analysis of *Porcn* mRNA in isolated $Porcn^{E\text{-}Del}$ intestinal crypts (N = 4) reveals reduction by orders of magnitude compared to controls (N = 2).

(D) Histology and immunohistochemistry (IHC) of $Porcn^{E\text{-}Del}$ (N = 5) and control ($Porcn^{+/Y}$;$Villin\text{-}Cre^{ERT2}$; N = 3) mice reveals no abnormalities. Left to right: hematoxylin and eosin (H&E) staining, Ki67 IHC, lysozyme IHC, and Alcian blue staining. High-magnification images are shown to the right of low-magnification views.

(E) Left, β-catenin IHC in control and $Porcn^{E\text{-}Del}$ mice shows nuclear-staining crypt-base cells (arrows). Right, qRT-PCR analysis of Wnt target mRNAs in isolated control (N = 2) and $Porcn^{E\text{-}Del}$ (N = 4) crypt epithelium demonstrates unperturbed Wnt-pathway activity.

Bars represent mean ± SEM of biological replicates; all scale bars, 50 μm.

$Villin^{-}$;$Myh11^{-}$ cells. Compound mutant mice showed no weight loss (Figure S1A) or clinical compromise, decrease in proliferation as assessed by Ki67 expression or bromodeoxyuridine (BrdU) uptake (Figures 3C and 3D), or defects in intestine morphology (Figures 3D, S1B, and S1C). Strong immunostaining for nuclear β-catenin provided direct evidence for sustained Wnt-signaling activity, and qRT-PCR analysis revealed subtle, statistically insignificant deficits in Wnt target transcripts (Figure 3E). Thus, combined loss of *Porcn* in the gut epithelium and the dominant

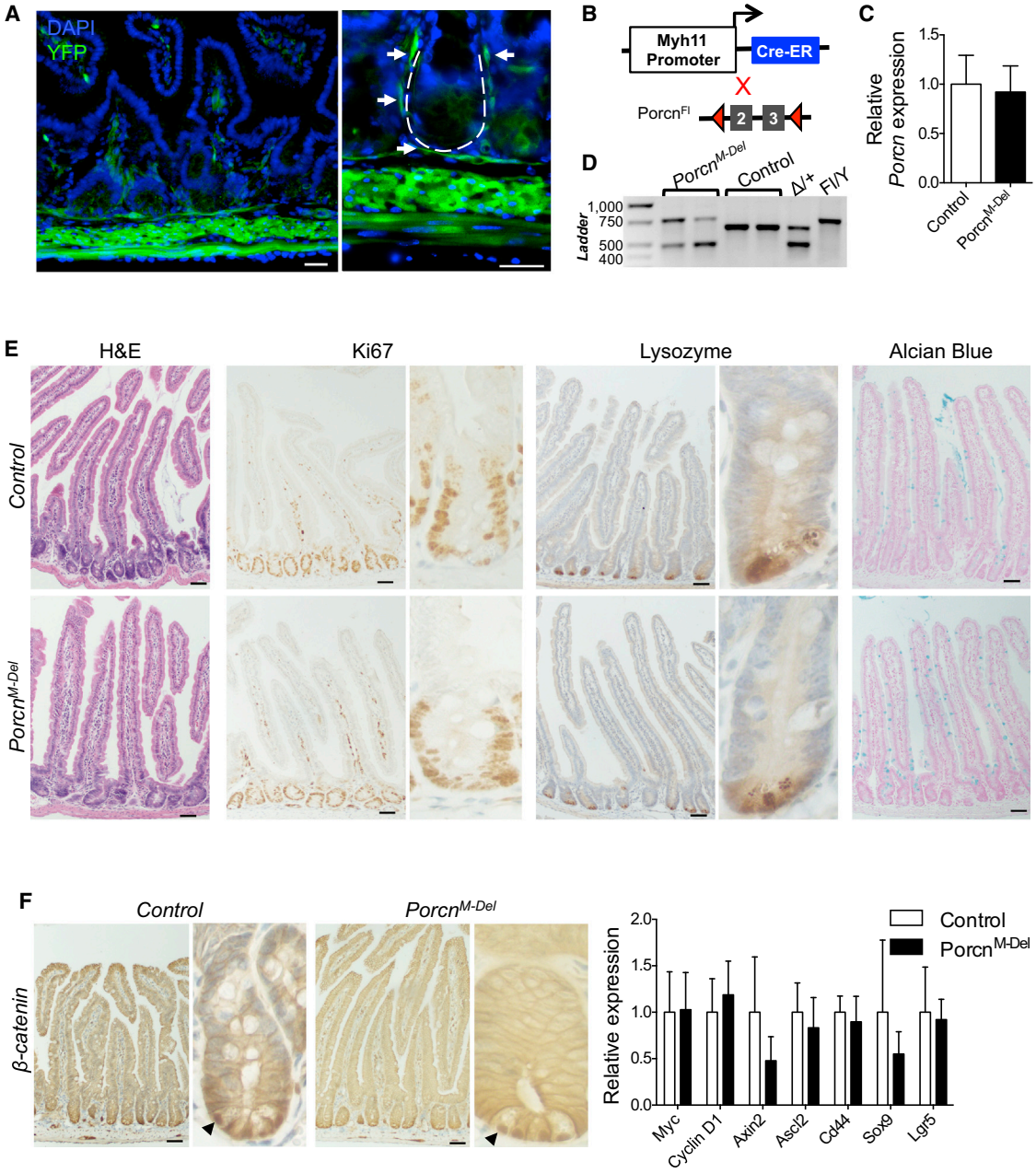

Figure 2. *Porcn* Loss in Intestinal Smooth Muscle Cells and SEMFs Does Not Adversely Affect Epithelial Morphology and Function

(A) *Myh11-Cre^{ERT2};Rosa26R* mice activate Cre recombinase in all muscle layers, including subepithelial myofibroblasts. The dotted line in the high-magnification image (right) outlines a crypt, and arrows point to the thin SEMF layer of YFP+ cells enveloping the crypt. The scale bars represent 30 μm. See also Figure S2.

(B) Strategy to induce muscle-cell-specific recombination in *Porcn$^{Fl/Y}$;Myh11-Cre^{ERT2}* mice.

(C) qRT-PCR analysis of isolated crypt epithelium shows no significant difference in *Porcn* mRNA expression in *Porcn$^{M\text{-}Del}$* (N = 4) mice compared to control (*Porcn$^{+/Y}$;Myh11-Cre^{ERT2}*, N = 4) mice, p = 0.35.

(D) PCR from whole-intestine genomic DNA, revealing the expected proportion of recombined *Porcn* in smooth muscle, with residual unrecombined *Porcn* DNA contributed by epithelial and other cell types that lack *Myh11-Cre^{ERT2}* expression.

(E) Histology and immunostains on *Porcn$^{M\text{-}Del}$* (N = 7) and control (*Porcn$^{+/Y}$;Myh11-Cre^{ERT2}*, N = 6) intestines reveal no abnormalities.

(F) Left, β-catenin IHC in control and *Porcn$^{M\text{-}Del}$* mice shows nuclear staining in crypt-base cells (arrows). Right, qRT-PCR reveals intact Wnt target expression in crypts isolated from *Porcn$^{M\text{-}Del}$* intestines, compared to controls (N = 4; statistics in Table S1).

Bars represent mean ± SEM of biological replicates; scale bars (E and F), 50 μm.

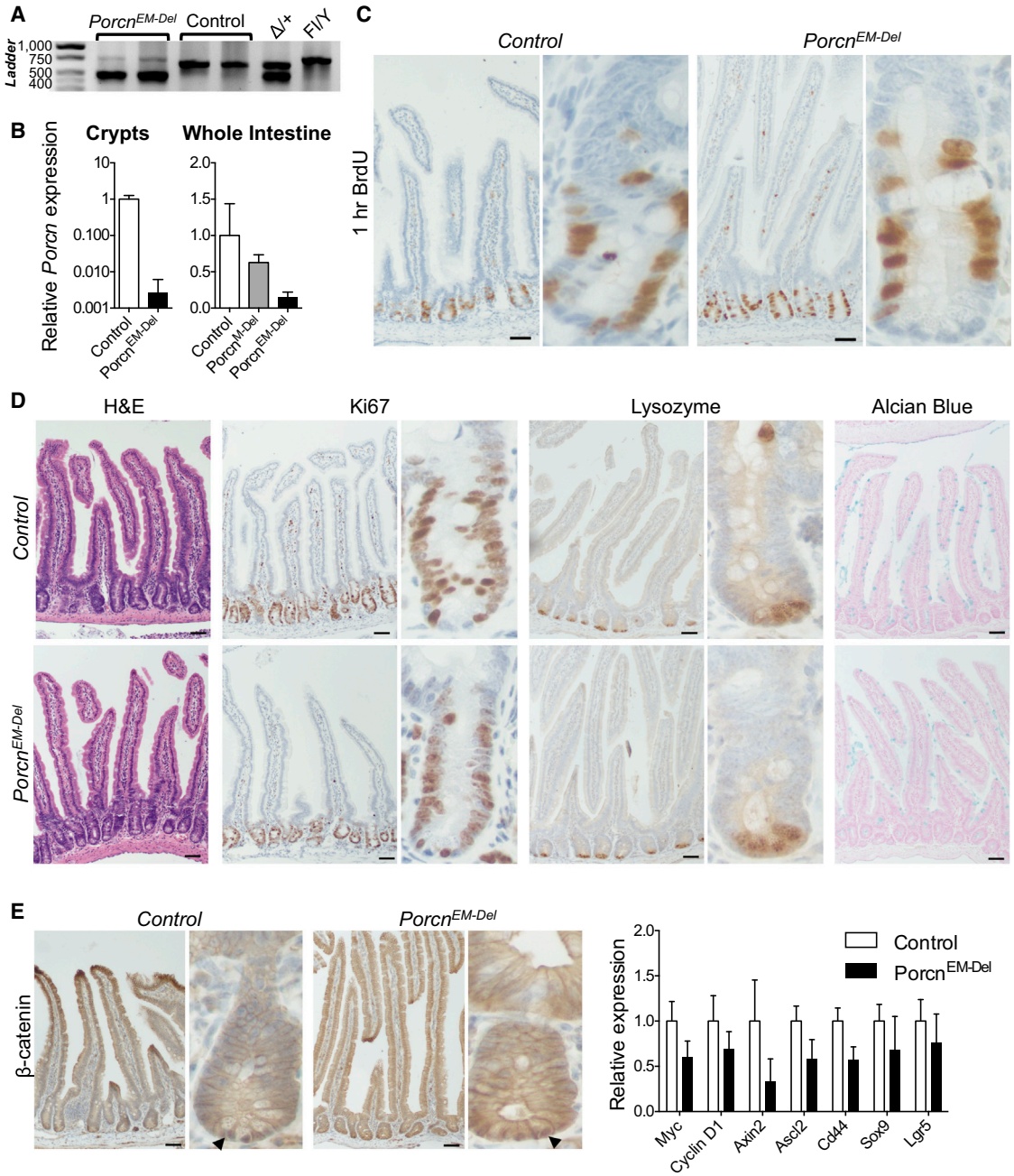

Figure 3. Consequences of Simultaneous Loss of *Porcn* from Epithelial and Muscle Cells

(A) DNA from whole intestines in TAM-treated *Porcn*$^{Fl/Y}$;*Villin-Cre*^{ERT2};*Myh11-Cre*^{ERT2} mice shows significant recombination at the floxed *Porcn* allele, with a minimal contribution of unrecombined DNA from nonepithelial, nonmuscle cells.

(B) Left, qRT-PCR analysis of RNA from crypts of *Porcn*$^{EM\text{-}Del}$ mutants (N = 3) compared to controls (*Porcn*$^{+/Y}$;*Villin-Cre*^{ERT2};*Myh11-Cre*^{ERT2}; N = 3) shows total *Porcn* deficiency, p = 0.0013. Right, qRT-PCR of unfractionated *Porcn*$^{M\text{-}Del}$ (N = 2) intestines shows lower *Porcn* expression than controls (*Porcn*$^{+/Y}$;*Villin-Cre*^{ERT2};*Myh11-Cre*^{ERT2} and *Porcn*$^{+/Y}$;*Myh11-Cre*^{ERT2}; N = 4), which is further reduced in *Porcn*$^{EM\text{-}Del}$ (N = 2) mice.

(C) Equivalent BrdU uptake in control and *Porcn*$^{EM\text{-}Del}$ intestinal crypts.

(D) Histology and immunostains from *Porcn*$^{EM\text{-}Del}$ and control intestines reveal intact morphology, cell proliferation, and differentiation (N = 3).

(E) Left, β-catenin IHC in control and *Porcn*$^{EM\text{-}Del}$ mice shows nuclear staining in crypt-base cells (arrows). Right, qRT-PCR analysis of control (N = 3) and *Porcn*$^{EM\text{-}Del}$ (N = 3) intestines indicates a statistically insignificant (Table S1) reduction in levels of Wnt target transcripts.

Bars in graphs represent mean ± SEM of biological replicates; all scale bars, 50 μm.

subepithelial compartment, including SEMFs, preserves all measurable Wnt-dependent functions.

These findings conflict with previous results from forced expression of Wnt antagonists, which produced crypt atrophy, villus shortening, loss of Wnt target transcripts, and failure of secretory cell differentiation within 2–4 days (Kuhnert et al., 2004). There are several possible reasons for the absence of similar defects in $Porcn^{Del}$ intestines. First, Wnts necessary for intestinal homeostasis might circumvent the requirement for PORCN in secretion. This is unlikely because two independent studies reveal that PORCN seems necessary to palmitoylate all human Wnts; this in turn is required for Wnts to bind the carrier protein Wntless for secretory transport (Coombs et al., 2010; Liu et al., 2013) and to bind Frizzled receptors (Janda et al., 2012; Najdi et al., 2012). Second, the potent effect of Wnt antagonists on crypt functions might not reflect the native activity of Wnts per se but rather of R-spondin or another family of ligands. Available evidence, however, indicates that R-spondins act in conjunction with, and not separate from, Wnts (Niehrs, 2012). A third possibility is that Wnt reserves in the subepithelial basement membrane or elsewhere persisted for the duration of our experiments. Wnts can bind heparan sulfate proteoglycans present on the surface of Wnt-recipient cells, a proposed mechanism to prevent their diffusion and allow prolonged activity (Mikels and Nusse, 2006). However, even in the absence of information on intestinal Wnt concentrations, turnover, or reserves, secreted Wnts are unlikely to have persisted for the length of our studies. *Porcn* deletion was efficient, and we deliberately examined mice weeks after Cre activation, giving time for Wnt reserves to decay. Moreover, in a tissue that self-renews continually and responds quickly to injury or cell loss (Clevers, 2013), Wnts probably turn over rapidly to allow responsive homeostasis.

We therefore favor the final possibility: that a cell type that evaded Cre-mediated *Porcn* deletion is a sufficient source of essential, intestine-active Wnts. That cell is unlikely to reside in the epithelium, where *Villin-*Cre^{ERT2} mice drove efficient Cre expression, leaving no intact *Porcn* DNA in isolated epithelial cells. By contrast, although *Myh11*-expressing smooth-muscle cells or SEMF are not a required physiologic Wnt source, gut mesenchyme contains diverse additional cell types, including endothelium, nonmuscle fibroblasts, leukocytes, lymphocytes, and neurons (Powell et al., 2011). Endothelial and neuronal contributions cannot readily be assessed in mice because their deficiencies are lethal early (Dumont et al., 1994; Enomoto et al., 1998), and intestinal functions seem intact in *Rag2*-null mice (Shinkai et al., 1992), indicating that lymphocytes either provide no essential Wnts or act redundantly with other cells. This extent of specificity or redundancy in intestinal Wnt source(s) challenges the prevailing view and reveals unanticipated complexity in control of intestinal self-renewal.

EXPERIMENTAL PROCEDURES

Mice

Villin-Cre^{ERT2}, *Myh11-*Cre^{ERT2}, *Rosa26-lox-STOP-lox-YFP*, and $Porcn^{Fl/Y}$ mouse strains were described previously (Barrott et al., 2011; el Marjou et al., 2004; Srinivas et al., 2001; Wirth et al., 2008). To induce recombination of conditional alleles, mice were injected intraperitoneally with 1 to 2 mg TAM (Sigma) dissolved in sunflower oil (Sigma) on 5 consecutive days. All animal care and procedures were approved and monitored by an Institutional Animal Care and Use Committee.

Tissue Harvests

Small intestines were dissected and flushed with cold PBS. The proximal and distal 1/3 were fixed overnight in 4% paraformaldehyde and then washed in PBS and embedded in paraffin or frozen for immunohistochemical analysis. The first 1 cm of the middle 1/3 was reserved for whole intestine (unfractionated) DNA or mRNA analysis. The remainder was used for isolating epithelium by incubating in 5 mM EDTA, shaking by hand, and passage through 70 μm filters to separate crypts and villi.

DNA Isolation and Genotyping

Epithelium or whole intestine was agitated in SNET buffer (0.2% SDS, 0.2 M NaCl, 100 mM Tris [pH 8], and 5 mM EDTA) with 15 μg Proteinase K at 55°C for 2 hr (epithelium) or overnight (whole intestine), and DNA was isolated. *Porcn* gene recombination was assessed using primers specifically detecting the wild-type, floxed, and deleted allele, as described in Supplemental Information.

RNA Isolation and Gene-Expression Analysis

RNA was isolated using TRIzol reagent (Invitrogen) and RNeasy mini kits (QIAGEN) followed by treatment with DNaseI and reverse transcription using the Superscript III First-Strand Synthesis System (Invitrogen). Quantitative RT-PCR was performed using FastStart Universal SYBR Green Master (Roche) and gene-specific primers; data analysis is described in Supplemental Information.

Histochemistry and Immunohistochemistry

Some mice received 1 mg BrdU (Sigma) 1 hr before euthanasia. Five-micrometer paraffin tissue sections were stained with hematoxylin and eosin, Alcian blue, or specific antibodies (Abs) to Ki67, BrdU, Chromogranin A, β-catenin, cleaved caspase 3, and lysozyme. Ten-micrometer frozen tissue sections were stained with smooth muscle actin Ab, followed by incubation with Alexa546-conjugated anti-immunoglobulin G. See Supplemental Information for antibody sources and conditions.

Detailed descriptions of all materials and methods can be found in the Supplemental Information.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, two figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.stemcr.2013.12.012.

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