Developmental Biology 391 (2014) 89–98

Contents lists available at ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/locate/developmentalbiology

Distinct requirements for beta-catenin in pancreatic epithelial growth and patterning

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ARTICLE INFO

Article history:
Received 26 January 2013
Received in revised form
26 March 2014
Accepted 27 March 2014
Available online 8 April 2014

Keywords:
Pancreas
Beta-catenin
Beta-cells
Progenitors
Notch
Islet

ABSTRACT

Pancreatic exocrine and endocrine lineages arise from multipotent pancreatic progenitor cells (MPCs). Exploiting the mechanisms that govern expansion and differentiation of these cells could enhance efforts to generate β-cells from stem cells. Although our prior work indicates that the canonical Wnt signaling component β-catenin is required qualitatively for exocrine acinar but not endocrine development, precisely how this requirement plays out at the level of MPCs and their lineage-restricted progeny is unknown. In addition, the contribution of β-catenin function to β-cell development remains controversial. To resolve the potential roles of β-catenin in development of MPCs and β-cells, we generated pancreas- and pre-endocrine-specific β-catenin knockout mice. Pancreas-specific loss of β-catenin produced not only a dramatic reduction in acinar cell numbers, but also a significant reduction in β-cell mass. The loss of β-cells is due not to a defect in the differentiation of endocrine precursors, but instead correlates with an early and specific loss of MPCs. In turn, this reflects a novel role for β-catenin in maintaining proximal–distal patterning of the early epithelium, such that distal MPCs resort to a proximal, endocrine-competent "trunk" fate when β-catenin is deleted. Moreover, β-catenin maintains proximal–distal patterning, in part, by inhibiting Notch signaling. Subsequently, β-catenin is required for proliferation of both distal and proximal cells, driving overall organ growth. In distinguishing two distinct roles for β-catenin along the route of β-cell development, we suggest that temporally appropriate positive and negative manipulation of this molecule could enhance expansion and differentiation of stem cell-derived MPCs.

Introduction

The adult pancreas can be divided into two functionally distinct domains, the exocrine function constituted by enzyme-secreting acinar cells and ducts, and the endocrine function performed by the islets of Langerhans. The division between the two domains begins shortly after specification where the pancreatic epithelium is separated into a distal "tip" domain at the periphery of the organ, which contains multipotent pancreatic progenitors (MPCs), and a proximal trunk domain, which contains bipotent progenitors that give rise to islet and duct cells. The capability of MPCs to produce all pancreatic lineages lasts roughly until the secondary transition at E13.5, after which distal tips are restricted to an acinar fate (Pan and Wright, 2011; Zhou et al., 2007). The early specification, maintenance and self-renewal of embryonic progenitors is critical to the development of the adult organ, as evidenced by the generation of a dysfunctional and smaller pancreas when early progenitors are partially ablated (Stanger et al., 2007).

With an ultimate goal of generating β-cells from stem cells, much effort has gone into understanding the transcription factors and intercellular signals that control pancreas progenitor patterning, expansion and differentiation (Pan and Wright, 2011). As signal transduction pathways mediate the response of progenitors to their microenvironment, they might serve as facile targets for manipulating stem cell differentiation. Nonetheless, while metazoan development requires only a few core signaling pathways, these can be deployed in complex and changing ways during organogenesis. This is exemplified, in the pancreas, by recent studies of the Notch signaling pathway. Originally implicated specifically as a negative regulator of endocrine differentiation (Apelqvist et al., 1999; Jensen et al., 2000), Notch has since been shown to inhibit acinar development as well (Esni et al., 2004; Hald et al., 2003; Murtaugh et al., 2003), potentially through modulating proximal–distal patterning of the early organ (Afelik et al., 2012; Magenheim et al., 2011; Schaffer et al., 2010). Although much has been gleaned from studying Notch and other pathways individually, relatively little is known about these signals coalesce to pattern and specify progenitors in the developing pancreas.

The goal of the present study is to elucidate the cellular mechanisms by which β-catenin, a component of the Wnt signaling pathway, controls the allocation of endocrine and exocrine lineages from pancreatic progenitor cells. In previous studies using

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http://dx.doi.org/10.1016/j.ydbio.2014.03.019
0012-1606/

two different "*Pdx1-Cre*^{early}" transgenic mice, both driving recombination in the early embryonic pancreas, conditional β-catenin knockout pancreata were found to be drastically smaller than wild-type and largely devoid of exocrine acinar cells, while containing a qualitatively normal complement of endocrine α- and β-cells (Murtaugh et al., 2005; Wells et al., 2007). However, deleting β-catenin using a different "*Pdx1-Cre*^{late}" transgene, beginning several days later in development (Heiser et al., 2006), produced little effect on acinar cell development but caused a mild decrease in islet cell mass (Dessimoz et al., 2005). It was hypothesized in that study that β-catenin was required separately for proliferation or survival of endocrine cells and exocrine progenitors. The discordant results of these studies may reflect the efficiency and timing of the different *Pdx1-Cre* deletor strains used (Murtaugh, 2008).The requirement for β-catenin in acinar cells persists through adulthood, when it is necessary for steady-state turnover and acinar cell regeneration following injury (Keefe et al., 2012). The role of Wnt/β-catenin signaling in differentiated endocrine cells has been an area of debate, with some studies suggesting it promotes β-cell proliferation and function (Dabernat et al., 2009; Rulifson et al., 2007), and another indicating that it is dispensable for adult mouse β-cell proliferation (Keefe et al., 2012). Together, these studies suggest that the contribution of β-catenin to endocrine islet development remains to be unraveled. Using time- and lineage-specific deletion experiments, we sought to investigate the roles of β-catenin during embryonic pancreas development, particularly in establishing endocrine β-cell mass.

Materials and methods

Mice

All experiments were performed according to protocols approved by the University of Utah IACUC. We obtained several mouse strains from the Jackson Laboratory: floxed and germline β-catenin loss-of-function mice (*Ctnnb1*$^{tm2Kem/J}$ and *Ctnnb1*$^{tm2.1Kem}$, henceforth *Ctnnb1*^{lox} and *Ctnnb1*$^{\Delta}$, respectively) (Brault et al., 2001); *Ngn3-Cre* BAC transgenic mice (Schonhoff et al., 2004) and the Cre-dependent EYFP reporter strain *Gt(ROSA)26Sor*$^{tm1(EYFP)Cos}$ (Srinivas et al., 2001), henceforth *R26R*^{EYFP}. *Pdx1-Cre* and *Pdx1-CreERT* transgenic mice (Gu et al., 2002) were provided by Doug Melton (Harvard University). To induce recombination with the *Pdx1-CreERT* transgene, we administered tamoxifen (Sigma) suspended in corn oil (Sigma) to pregnant dams, typically 8–16 weeks of age, by oral gavage. Embryos were genotyped by PCR, using primer sets described previously (Gu et al., 2002; Murtaugh et al., 2005).

Tissue processing and staining

Pregnant dams were euthanized with isoflurane followed by cervical dislocation. Whole embryos (E13.5 and younger) and pancreata (E14.5 and later) were dissected in ice-cold PBS for processing. Tissues were fixed overnight at room-temperature with zinc-buffered formalin (Polysciences) for paraffin sections or with 4% paraformaldehyde/PBS (2 h-overnight at 4 °C for frozen sections, and further processed as previously (Keefe et al., 2012; Kopinke and Murtaugh, 2010; Murtaugh et al., 2005)). A series of duplicate paraffin sections (6 μm) were collected sequentially across multiple slides, spaced with skipping to span the entire pancreas in the following age-dependent manner: for E17.5, 10 slides with 180 μm between sections; E12.5–E14.5, 8 slides with 96 μm between sections; E11.5, 7 slides with 84 μm between sections on slides. In this way, the entire volume of each pancreas is sample on multiple individual slides. Similarly, frozen sections (8 μm) were collected serially over 6–10 slides such that the each slide contained representative sections throughout the organ. For labeling S-phase nuclei, mice were injected with BrdU (50 μg/g body weight) one hour prior to sacrifice.

Antibodies used for immunostaining are listed in Table S1, and all secondary antibodies (raised in donkey) were purchased from Jackson Immunoresearch. Immunostaining was performed as previously (Keefe et al., 2012; Kopinke and Murtaugh, 2010; Murtaugh et al., 2005), including high-temperature antigen retrieval for paraffin sections. For anti-BrdU staining, frozen sections were pre-treated with DNase I (700 u/μl, in 40 mM Tris–HCl pH 7.4, 10 mM NaCl, 6 mM $MgCl_2$, and 10 mM $CaCl_2$) at room temperature for 30 min (Ye et al., 2007). Bright field images were obtained using an Olympus CX41 microscope and MicroSuite software. For immunofluorescence, Fluoromount-G (Southern Biotech) was used as a mounting substrate and images were obtained using an Olympus IX71 microscope and MicroSuite software. Identical exposure times and post-processing adjustments performed in Adobe Photoshop were used across control and experimental genotypes.

Quantification and analysis

To measure β-cell mass, pancreas size, and volume or number of cells expressing various markers, serial sections were stained by immunohistochemistry, and all sections on a single slide were photographed individually (4× original magnification at E17.5, and 10–20× for all other time points), to provide a representation of the entire pancreas. ImageJ (NIH) software was used to measure the surface area occupied by stained tissue. For Ptf1a, c-Myc, and Ngn3 at E11.5 and E12.5, stained nuclei were counted in Adobe Photoshop. The total number of cells per pancreas (E11.5–E13.5) was estimated by multiplying the number of cells counted per slide by the number of slides in the series. Calculations, graphs and *P*-values (two-tailed, unpaired *t*-test) were generated in Microsoft Excel, and results are presented as mean ± s.e.m.

For lineage tracing experiments using *Pdx1-CreERT* and *R26R*^{EYFP}, we photographed several independent fields (20× original magnification), per embryo, across multiple pancreatic sections per slide. For acinar cell labeling indices, we used the additive image overlay feature of ImageJ (NIH) software to identify the overlap of DAPI, amylase (acinar cells) and EYFP, and counted cells using the Analyze Particles function (Kopinke and Murtaugh, 2010). For all other differentiation markers, cells were counted using the Count Tool in Adobe Photoshop.

Explant cultures and wholemount immunostaining

For ex vivo explant cultures, the dorsal buds of E11.5 pancreata were dissected in ice-cold sterile PBS, and cultured at the air-media interface on 0.4 μm pore size PTFE cell culture inserts (Millipore), in DMEM with 10% fetal bovine serum and antibiotics. A small piece of tissue was collected for genotyping purposes at the time of dissection. Explants were treated with 100 nM of the γ-secretase inhibitor DBZ (Millipore 565789) for 3 days, while controls received no treatment. Media was changed daily.

Wholemount immunofluorescence was performed as previously described (Kopinke and Murtaugh, 2010). Briefly, explants were fixed overnight in 4% PFA, washed and stored in methanol until staining. For staining, explants were rehydrated to PBS, permeabilized for 1 h with 1% Triton-X100 in PBS, and then placed in blocking solution (5% donkey serum and 0.3% Triton X-100) for 2 h. Primary and secondary antibody incubations were performed overnight at room temperature. Explants were cleared in BABB (2:1 benzyl alcohol:benzyl benzoate) prior to imaging. Confocal images were obtained using an Olympus FV-1000 microscope.

Results

Both pancreas size and beta-cell mass depend on β-catenin function in multipotent progenitor cells

As in our previous study (Murtaugh et al., 2005), we used the *Pdx1-Cre* early transgene (henceforth, *Pdx1-Cre*) (Gu et al., 2002; Heiser et al., 2006) to delete the conditional *Ctnnb1*^{lox} allele (Brault et al., 2001), producing pancreas-specific β-catenin knockout (PBKO) mice (Fig. 1A). Confirming published results, we found that mutant pancreata were drastically smaller at late fetal stages, due mostly to the loss of exocrine acinar cells (Fig. 1B and C). To more accurately assess the effect of deleting β-catenin, we developed a strategy of absolute volume quantification, in which evenly-spaced sections were collected across a series of slides, to capture the entire pancreas (see Materials and methods). Integrating the area occupied by immunostaining for individual markers allowed us to estimate the absolute volume of pancreas occupied by specific cell types. Staining for the pan-epithelial marker E-cadherin at E17.5 revealed a 10-fold reduction in overall pancreas size in PBKO compared to controls (Fig. 1D–F). The drastic reduction in size is mostly due to the loss of exocrine acinar cells as shown previously (Murtaugh et al., 2005; Wells et al., 2007), together with a decrease in duct cells (Fig. 1J). Casual observation suggested a possible increase in β-cell numbers in PBKO, as β-cells occupied more of the remnant epithelium than in control pancreata. However, quantification revealed a 2-fold reduction in β-cell volume following β-catenin deletion, not documented in any previous study (Fig. 1G–I). The volume of other endocrine cell types, including α-cells and δ-cells, exhibited a similar reduction, representing a deficit in overall islet cell mass (Fig. 1J). Importantly, the size of β-cells was indistinguishable between wild-type and mutant (Fig. S1A–C), confirming that the decrease in β-cell volume reflected a decrease in β-cell numbers.

Because *Pdx1-Cre*^{early} deletes β-catenin in early pancreatic progenitor cells, analysis of late fetal PBKO pancreata cannot distinguish between defects that arise early, in time or lineage progression, from those arising late. We were particularly interested in establishing if the decreased β-cell mass in PBKO reflected a requirement for β-catenin in insulin+ cells themselves and/or in Ngn3+ endocrine precursors. We used an *Ngn3-Cre* BAC transgenic mouse line (Schonhoff et al., 2004) to delete *Ctnnb1* specifically in Ngn3+ precursors and their progeny, avoiding any potential effect in Pdx1+ MPCs. In contrast to *Pdx1-Cre*^{early}-mediated deletion, we found no change to β-cell mass following deletion with *Ngn3-Cre* (Fig. 1K–M). These data suggest that β-catenin acts at a stage prior to endocrine specification to establish both normal pancreas size and normal β-cell mass.

To determine the specific period in which β-catenin was required during development, we compare wild-type and mutant pancreas size at several developmental time points. Although pancreas size was not significantly affected at E11.5, β-catenin deficient pancreata were significantly smaller than control beginning at E12.5, the discrepancy increasing with time (Fig. 2). Interestingly, the timing of the PBKO pancreas hypoplasia phenotype corresponds approximately to the classically-described "secondary transition" (Pan and Wright, 2011; Pictet and Rutter, 1972), defined by an exponential increase in the numbers of differentiated

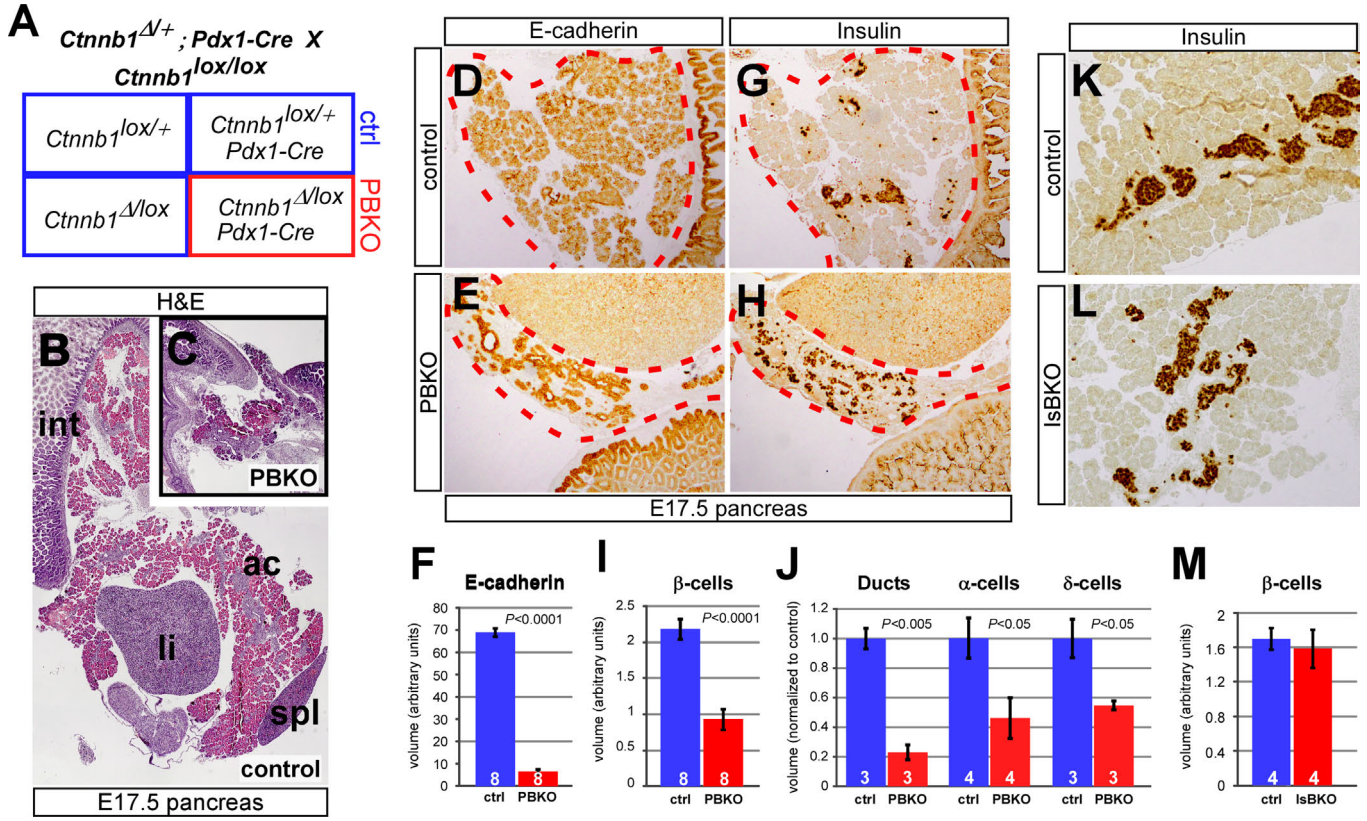

Fig. 1. β-Catenin is required for pancreas and β-cell mass prior to endocrine specification. *Pdx1-Cre* (PBKO) or *Ngn3*-Cre (IsBKO) β-catenin knockout pancreata were analyzed and compared to controls at E17.5. (A) Breeding scheme to generate control and KO genotypes. (B and C) H&E staining of E17.5 pancreata reveals a dramatic loss of acinar tissue (pink) in PBKO compared to control. (D–F) Pancreata (red-dashed outline) stained with the pan-epithelial marker E-cadherin to determine total pancreas size exhibit a 10-fold reduction in PBKOs compared to controls. (G–I) Although insulin+ β-cells occupy a greater proportion of PBKO pancreatic epithelia (adjacent sections to D and E), quantification reveals a 2-fold reduction in absolute β-cell volume. (J) Quantification of ducts (DBA-lectin+), α-cells (glucagon+), and δ-cells (somatostatin+), normalized to controls at E17.5, demonstrates that PBKOs have reduced exocrine and endocrine numbers. (K and M) IsBKO (*Ngn3-Cre)* and control pancreata show no difference in β-cell volume at E17.5. The number of embryos analyzed per genotype is indicated at the bottom of each bar.

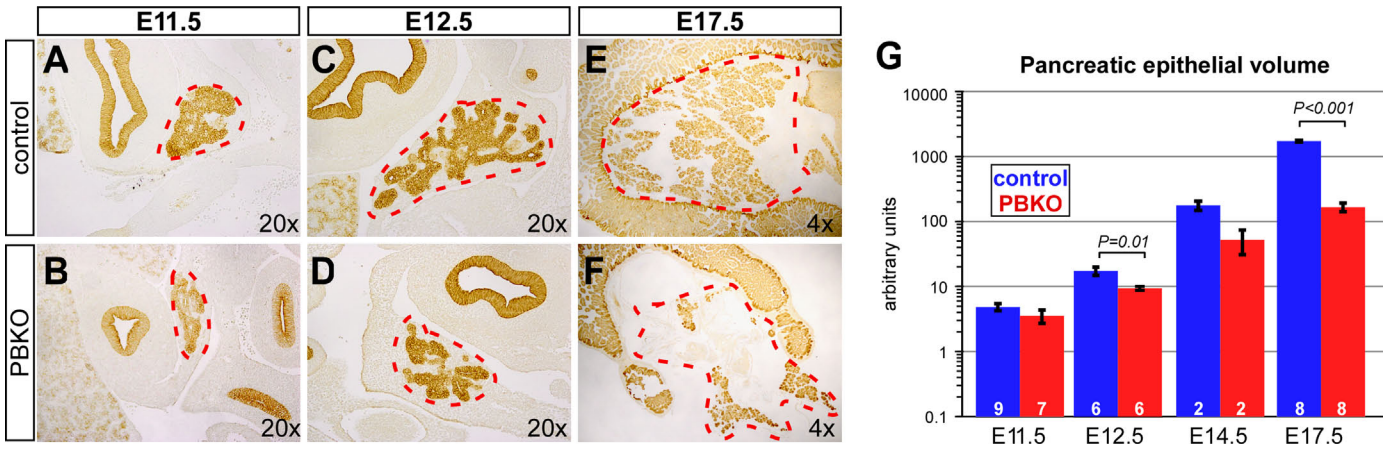

Fig. 2. Progressive growth defect in β-catenin mutant pancreata. PBKO or control pancreata from E11.5, E12.5 and E17.5 were stained with the pan-epithelial marker E-cadherin to determine overall pancreas size (dashed outline). (A and B) At E11.5, pancreas size is similar between PBKOs and controls. (C and D) At E12.5, PBKO pancreata are smaller and contain fewer distal branches than controls. (E and F) Staining for the pan-epithelial marker E-cadherin at E17.5 indicates a dramatic deficiency of epithelial tissue in PBKOs compared to controls. Control and mutant images photographed at identical magnifications, as indicated. (G) Volume quantification of E-cadherin-stained surface area (graphed on the log axis) reveals a size deficit in PBKO pancreata beginning at E12.5, increasing through E17.5.

endocrine and exocrine cells. As our quantitative analysis identified E12.5 as the first stage at which phenotypic changes could be observed in PBKO, we focused our studies on this period to characterize and quantify changes to the MPC population.

Early loss of distal MPCs in β-catenin-deficient pancreata

Lineage tracing studies reveal that all mature pancreatic cell types arise from MPCs (Zhou et al., 2007). Following specification and prior to the secondary transition, the pancreatic epithelium segregates into a MPC-containing distal tip domain and a proximal trunk domain that comprises duct and endocrine precursors (Pan and Wright, 2011; Zhou et al., 2007). To characterize any changes to the distal MPC population, we stained and quantified potential MPCs at E12.5, labeled independently with three markers, Ptf1a, carboxypeptidase 1 (Cpa1) and c-Myc (Zhou et al., 2007). We found MPC numbers to be dramatically reduced in PBKO, regardless of the marker used (Fig. 3A–C, D–F, and G–I). This reduction is not simply explained by the overall decrease in PBKO epithelial volume, as the proportion of Ptf1a+ cells within the pancreas was also dramatically reduced (Fig. S2A). The remaining Ptf1a+ cells found in PBKO pancreata likely represent "escaper" cells that fail to delete β-catenin, and later provide the only source of residual acinar cells in these embryos (Murtaugh et al., 2005). Overall, the morphology of the PBKO epithelium resembled a proximal trunk domain devoid of branching tips.

In addition to staining for distal tip MPCs, we looked for any potential defects within the proximal and pre-endocrine populations by staining for Sox9 or Ngn3, respectively. At E12.5, Sox9 is still expressed in both the distal and proximal domain, and we observed no change in the Sox9 expression pattern (Fig. S3). However, in contrast to the loss of distal MPCs, we found an unexpected increase of Ngn3+ cells, both in absolute number and as a proportion of the trunk epithelium (Figs. 3J–L and S2B). Furthermore, some Ngn3+ cells remained in the distal domain one day later, at E13.5 (data not shown). Consistent with the bias of early Ngn3+ cells toward the α-cell lineage (Johansson et al., 2007), we found a modest increase in glucagon+ cells in PBKOs at this time, suggesting that extra Ngn3+ cells present at E12.5 in PBKOs had differentiated (Fig. S4). Given that acinar cells arise from distal tip MPCs persisting at the secondary transition (Zhou et al., 2007), our data suggest that the acinar deficit of PBKO mice reflects an earlier defect in maintenance of distal tip identity, such that β-catenin-deficient distal MPCs take on proximal "trunk" cell characteristics.

Pancreatic patterning is established but not maintained in PBKO

Given that β-catenin-deficient pancreata contained fewer distal MPCs at E12.5 than controls, we sought to determine whether β-catenin was required for establishing or maintaining MPC identity and distal patterning. We identified MPCs at E11.5, a time point prior to any noticeable decrease in total epithelial size, based on their expression of Ptf1a or c-Myc (Zhou et al., 2007), and found no significant difference in the number of Ptf1a+ cells between controls and PBKOs (Fig. 4A–C). The number of c-Myc+ cells, however, was already reduced in E11.5 PBKOs compared to controls (Fig. 4D–F), similar to our findings at E12.5. Importantly, c-Myc is a β-catenin target gene (He et al., 1998) and known to promote pancreatic epithelial proliferation (Bonal et al., 2009; Nakhai et al., 2008), suggesting that its loss of expression reflected loss of β-catenin signaling activity.

Interestingly, the total number of Ptf1a+ cells present in E11.5 PBKOs was greater than the number at E12.5 (Fig. 4C), suggesting that the deficit observed at the later stage may reflect more than a simple failure to expand. Confirming previous results (Murtaugh et al., 2005), we found no detectable increase in cell death in PBKO pancreata at these stages (data not shown). We therefore interpret our results as indicating normal MPC specification at E11.5, followed by a progressive shift from distal to proximal fate of these cells in the absence of β-catenin function.

To test the hypothesis that β-catenin acts post-specification to support MPC maintenance and acinar development, we used the tamoxifen-inducible *Pdx1-CreERT* deletor (Gu et al., 2002) to achieve temporal control of *Ctnnb1* recombination. We also bred in the Cre-dependent *R26R*^{EYFP} reporter (Srinivas et al., 2001), allowing for lineage tracing of EYFP+/β-catenin-deficient cells. These mutant embryos are referred to as mPBKO, for mosaic pancreas-specific β-catenin knockout. We first administered tamoxifen to pregnant dams at E10.5 and harvested pancreata at E17.5, analyzing EYFP distribution to determine the fate of manipulated cells. Our previous study using constitutive *Pdx1-Cre* indicated that β-catenin-deficient cells are excluded from the acinar lineage while remaining competent for β-cell differentiation (Murtaugh et al., 2005). Consistent with this, we found a dramatic reduction in the acinar contribution of EYFP+ cells in mPBKO pancreata following E10.5 TM treatment, while β-cell

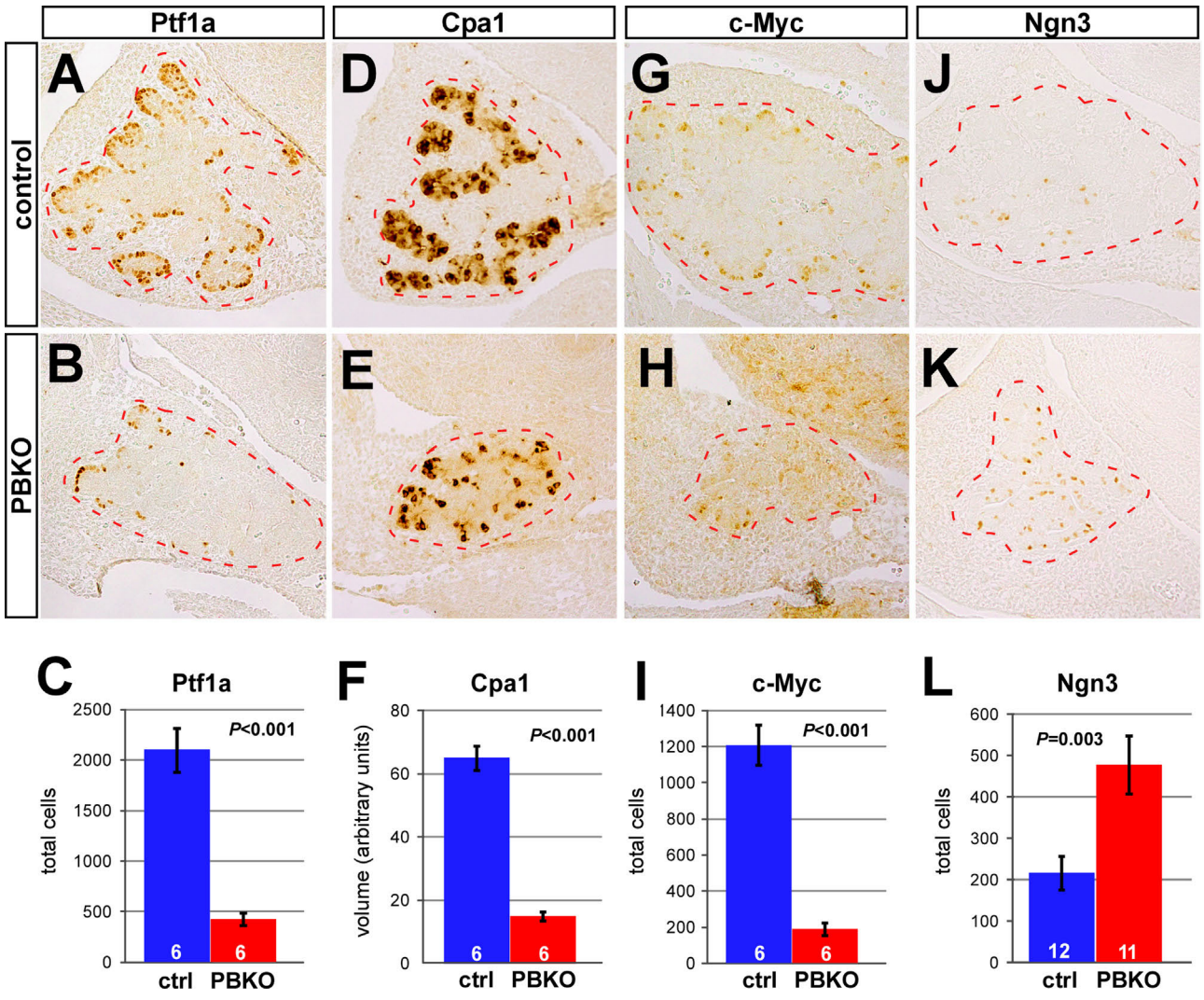

Fig. 3. Proximalization of β-catenin deficient pancreata during MPC expansion. E12.5 mutant and control pancreata were stained for Ptf1a, Cpa1 and c-Myc to label distal MPCs, and Ngn3 to label endocrine precursors (epithelia are outlined in red). (A–C) Control Ptf1a staining identifies MPCs localized to the distal tips of the expanding epithelium. PBKO pancreata contained fewer distal tips and fewer Ptf1a+ cells than age-matched controls, confirmed by quantification. (D–F) Similar to Ptf1a, the distal MPC marker Cpa1 exhibits a dramatic decrease in PBKOs compared to controls. (G–I) Cells expressing the Wnt target gene c-Myc are identified in distal tips of control but not PBKO pancreata. (J–L) Staining for the endocrine precursor marker Ngn3shows an absolute increase in Ngn3+ cells in PBKOs, with some Ngn3+ cells localizing abnormally to distal tips rather than being confined to the proximal trunk domain. Quantification of Ptf1a, c-Myc and Ngn3 is represented as the total number of cells calculated per pancreas.

labeling remained comparable to controls (Fig. S5). Our mosaic deletion/labeling approach thus provides independent evidence that β-catenin is required in the acinar lineage, and indicates that this requirement applies during stages of MPC expansion.

To examine more closely the fate of β-catenin deficient MPCs, we administered tamoxifen at either E8.5 (during/prior to MPC specification) or E10.5 (following specification), and harvested embryos 3 days later at either E11.5 or E13.5, respectively. In the E8.5–E11.5 pulse-chase paradigm, we found that Cpa1+ MPCs exhibited a similar EYFP labeling index in control and mPBKO (Fig. 5A–C), consistent with a lack of requirement for β-catenin in the initial generation of MPCs. Staining for β-catenin protein confirmed that labeled cells in mPBKO pancreata were β-catenin-deficient (Fig. 5B). In the E10.5–E13.5 paradigm, however, we found that Cpa1+ cells were labeled much less frequently in mPBKOs than in controls, and EYFP+ cells were generally excluded from distal tips and confined to proximal trunk regions (Fig. 5D–F). Moreover, we found that the majority of EYFP+ Cpa1+ cells in E10.5–E13.5 mPBKOs were “escapers” that had not successfully deleted *Ctnnb1*, as indicated by β-catenin staining (Fig. 5E). Together with the results of our PBKO studies, above, these lineage tracing experiments suggest that β-catenin is required for MPC maintenance and proximal–distal patterning, and acts during MPC expansion rather than specification.

β-Catenin regulates proliferation independent of proximal–distal patterning

Intuitively, if MPC numbers are determined by integrating their self-renewal with differentiation to a bipotent proximal fate (Zhou et al., 2007), then the progressive MPC depletion we observe in PBKO between E11.5 and E12.5 (Fig. 4C) could reflect a growth arrest of these cells. Indeed, Wnt/β-catenin signaling is well known to regulate proliferation in many tissues, including mature acinar cells (Keefe et al., 2012; Polakis, 2012; Schuijers and Clevers, 2012), and decreased proliferation has been documented in β-catenin mutant pancreata, albeit at isolated time points and without reference to specific cell types affected (Dessimoz et al., 2005; Murtaugh et al., 2005; Wells et al., 2007).

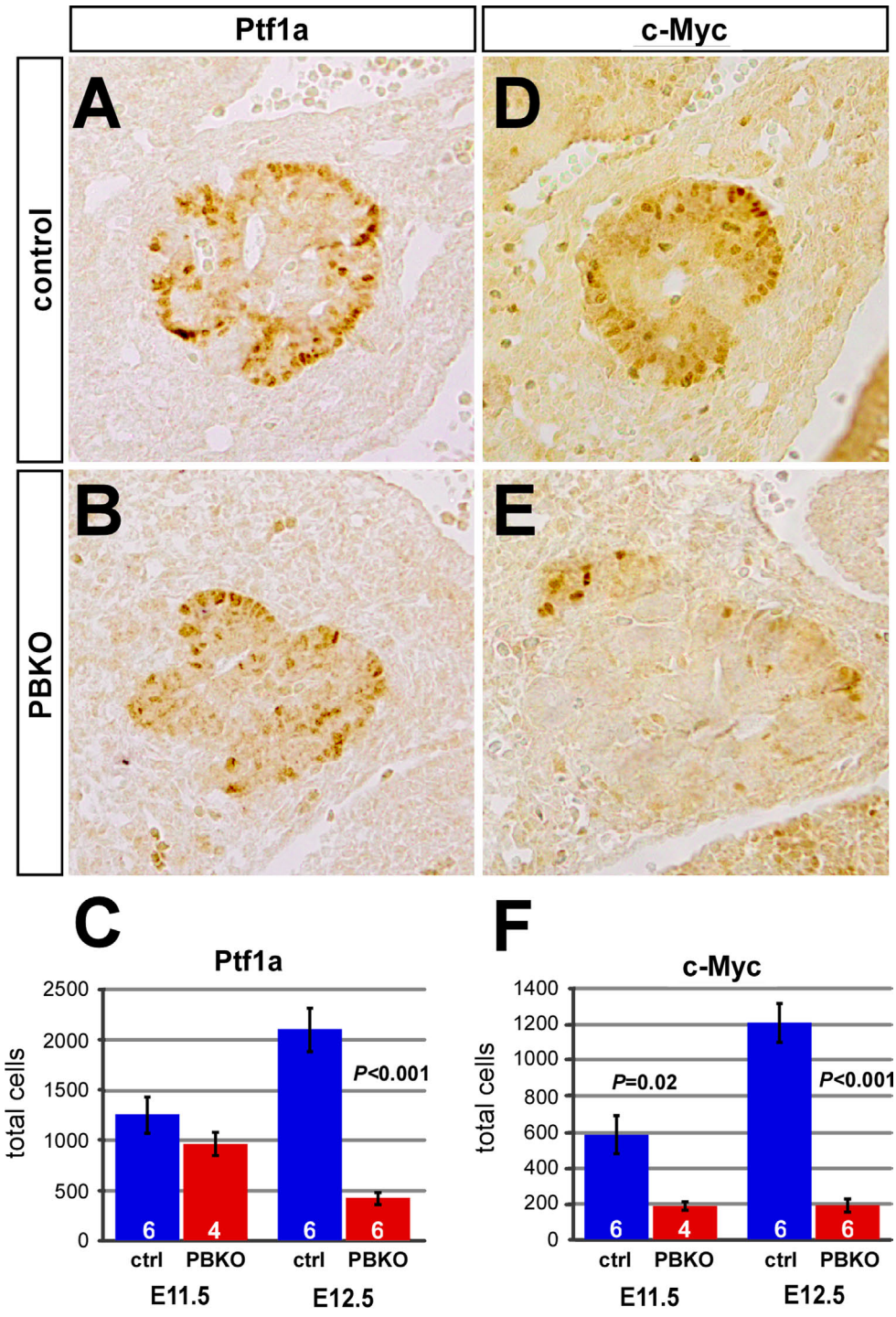

Fig. 4. Proximal–distal patterning is established in the absence of β-catenin. E11.5 pancreata were stained with Ptf1a and c-Myc to label and quantify distal MPCs. Total cell number indicates the number of labeled cells calculated per pancreas. (A and B) Ptf1a staining is similar between control and PBKO at this stage, with Ptf1a+ cells localized to the distal periphery. (C) Ptf1a+ cells at E11.5 show a non-significant difference between PBKO and control. The total number of Ptf1a+ cells (reproduced from Fig. 3C) increased in controls from E11.5 to E12.5, and decreased in PBKOs. (D and E) Staining for c-Myc reveals a decrease in the number of c-Myc+ cells in PBKOs compared to controls. (F) C-Myc+ cells are markedly decreased in E11.5 PBKO pancreata.

To determine the spatiotemporal requirements for β-catenin in MPC proliferation, we injected pregnant mice with BrdU to label proliferating cells one hour prior to harvest at E11.5, E12.5 and E13.5, and analyzed BrdU labeling of Cpa1+ distal MPCs. Surprisingly, we found similar levels of proliferation between controls and PBKOs at E11.5 (Fig. 6A–F). Beginning at E12.5 and continuing through E13.5, however, PBKO cells incorporated BrdU less frequently than age-matched controls (Fig. 6G–L and M–R). We calculated the percentage of BrdU+ cells among all pancreatic epithelial cells, marked in this experiment by activation of *R26R*^{EYFP}, as well as in Cpa1+ cells specifically, and found no significant difference in proliferation in either population at E11.5. Cpa1+ cell proliferation remained constant in controls through all periods, whereas overall proliferation begins to decline at E13.5. In PBKOs, both overall and Cpa1+ cell proliferation fell dramatically at E12.5 and remained reduced at E13.5 (Fig. 6S and T). With little or no proliferation defect in mutant MPCs at E11.5, their rapid disappearance over the next 24 h is most readily explained by a

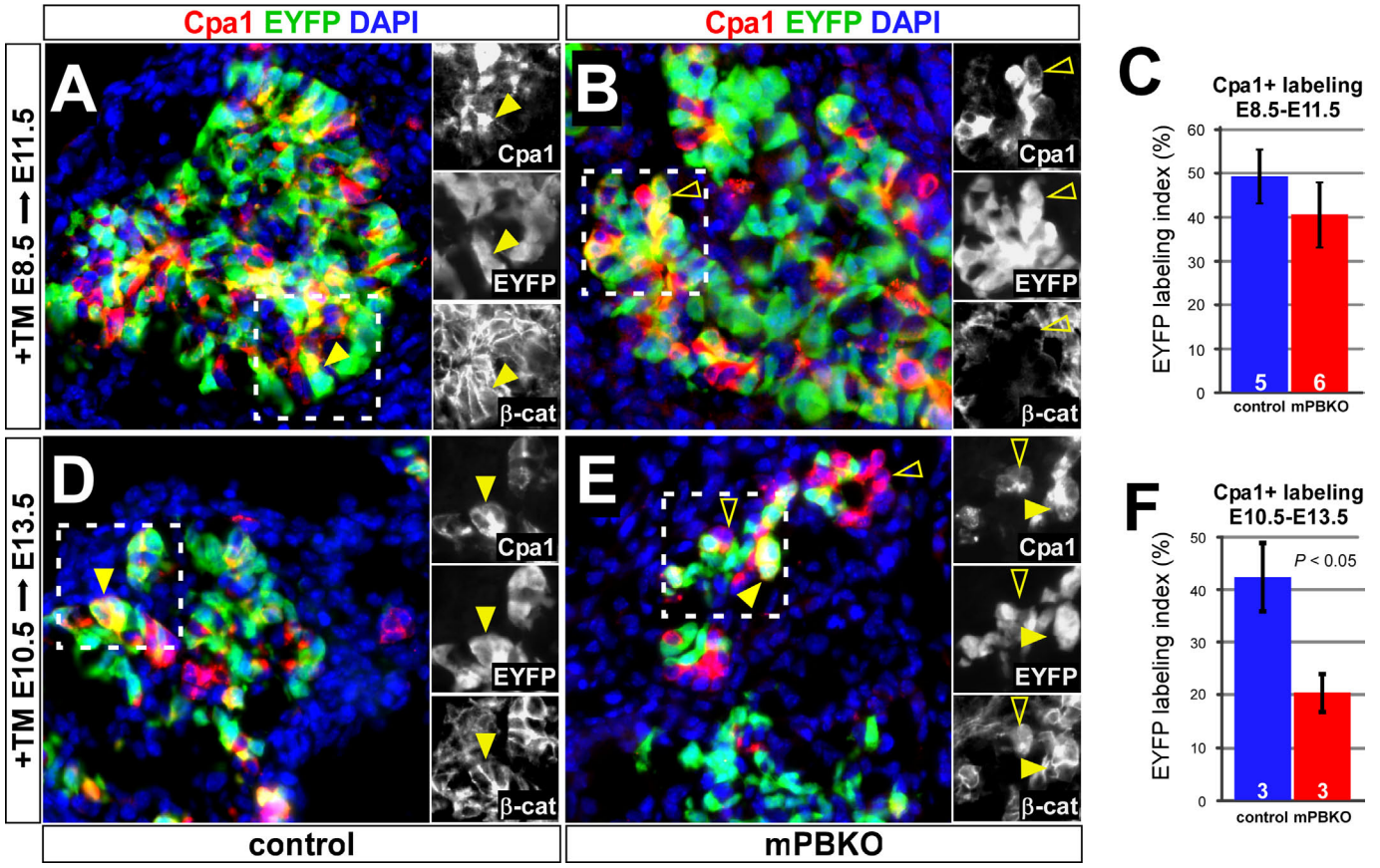

Fig. 5. β-Catenin is required to maintain but not establish distal tip cell identity. Control (*Ctnnb1*$^{lox/+}$; *R26R*$^{EYFP/+}$; and *Pdx1-CreERT*/+) and mosaic pancreas-specific β-catenin knockout (mPBKO: *Ctnnb1*$^{\Delta/lox}$; *R26R*$^{EYFP/+}$; and *Pdx1-CreERT*/+) embryos received tamoxifen in utero at either E8.5 or E10.5 and were analyzed 3 days later (E11.5 and E13.5, respectively) by immunofluorescence. (A) Cpa1+ distal cells (red) from control pancreata given tamoxifen at E8.5 and harvested at E11.5 co-express EYFP (green) as indicated by yellow arrowheads. Monochrome panels depict single color channels for Cpa1 and EYFP, from area highlighted in dashed box, together with β-catenin protein (not included in the merge, but taken from the same field), and confirm that β-catenin is expressed by EYFP+ Cpa1+ cells in control (closed arrowheads). (B) Cpa1+ cells (red) from mPBKO littermates co-express EYFP (green). Single color channels indicate that most EYFP-labeled Cpa1+ cells are β-catenin-deficient (open arrowheads). (C) The EYFP labeling indices of Cpa1+ cells remain similar between controls and mPBKO. (D) Cpa1+ distal tip cells from controls given tamoxifen at E10.5 and harvested at E13.5 co-express EYFP and β-catenin. (E) In mPBKO littermates, EYFP+ and β-catenin-deficient cells are generally excluded from distal Cpa1+ tips. Remaining Cpa1+ EYFP+ cells retain β-catenin expression, indicating a selection for MPCs that have not deleted β-catenin (closed arrowheads). Open arrowheads indicate unlabeled, β-catenin+ Cpa1+ cells that comprise a large majority of the Cpa1+ population. (F) After an E10.5 TM pulse, control Cpa1+ cells retain a higher EYFP labeling index than mPBKO.

key requirement for β-catenin in distalization of the epithelium. Our results suggest that the loss of MPCs, through altered patterning leading to premature differentiation as well as through reduced proliferation, ultimately diminishes influx into the pool of precursors that generates β-cells later in development (Fig. 7H).

Notch signaling is required for proximalization induced by loss of β-catenin

Elimination of β-catenin and elimination of Notch signaling have opposite patterning consequences in the developing pancreas: whereas loss of β-catenin produces a shift toward proximal/trunk patterning, the inhibition of Notch promotes distal/tip cell development (Afelik et al., 2012; Magenheim et al., 2011). Moreover, ectopic activation of Notch prevents acinar cell differentiation, similar to β-catenin loss-of-function (Esni et al., 2004; Hald et al., 2003; Murtaugh et al., 2003). Notch and Wnt/β-catenin have been found to have antagonistic effects in other experimental systems as well, although this appears to be context-specific (Acosta et al., 2011; Hayward et al., 2005; Kwon et al., 2011). To determine the relationship between these pathways in the pancreas, we cultured E11.5 PBKO pancreata in the absence or presence of the gamma-secretase inhibitor DBZ, which inhibits ligand-induced Notch receptor activation (Magenheim et al., 2011; Milano et al., 2004). After 3 days of culture, untreated PBKO explants developed few Cpa1+ tip/pro-acinar cells, most of which represented β-catenin+ "escaper" cells (Fig. 7A–C). DBZ-treated PBKO explants, however, developed large numbers of Cpa1+ cells within β-catenin deficient areas (Fig. 7D–F). Notably, the number of Cpa1+ escaper cells was not increased by DBZ, indicating that Notch inhibition did not induce generalize hyperplasia of tip/pro-acinar cells (Fig. 7G). These results provide insight into the mechanism by which β-catenin normally promotes distal fate, namely by inhibiting the ability of Notch to drive tip-to-trunk conversion (Fig. 7H).

Discussion

We previously found that the multifunctional protein β-catenin was required for pancreatic acinar development but dispensable for endocrine differentiation and function (Murtaugh et al., 2005). We have revisited the pancreas-specific β-catenin knockout model to reveal a requirement for β-catenin in generating normal islet cell numbers and, through the use of lineage-restricted Cre deletor lines, we demonstrate that this requirement is imposed prior to endocrine specification. β-catenin has two distinct roles in the early pancreas: it promotes distal patterning of the epithelium by inhibiting Notch, and it is required for maximal proliferation throughout the developing organ (Fig. 7). Our findings provide

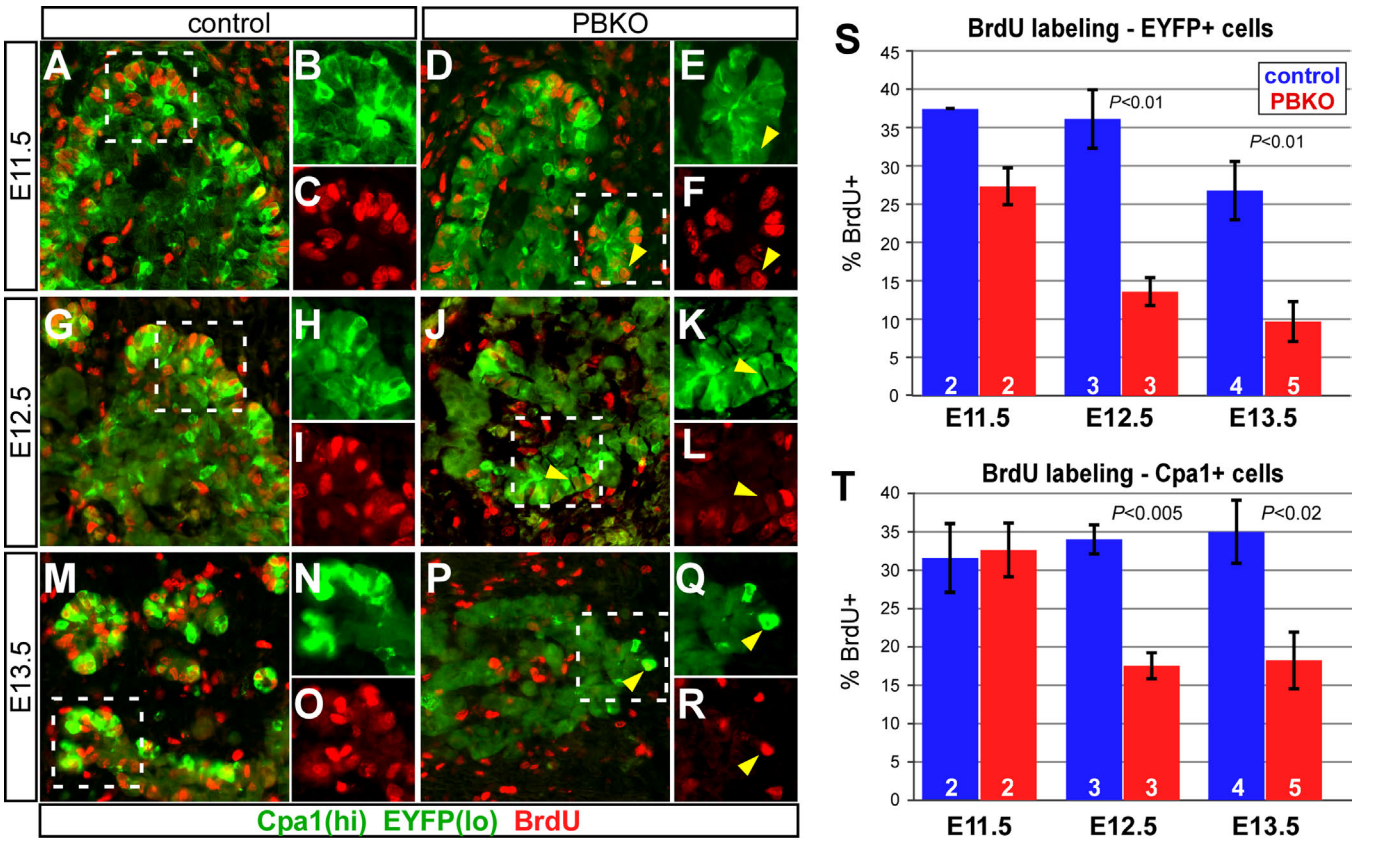

Fig. 6. β-Catenin is required after E11.5 for epithelial proliferation. Control (*Ctnnb1*$^{lox/+}$; *R26R*$^{EYFP/+}$; and *Pdx1-Cre/+*) and PBKO (*Ctnnb1*$^{\Delta/lox}$; *R26R*$^{EYFP/+}$; and *Pdx1-Cre/+*) littermates received BrdU in utero one hour prior to sacrifice at E11.5, E12.5 or E13.5, and proliferation index was assessed via BrdU immunostaining (red). All pancreatic epithelial cells are EYFP-labeled (light green) due to *Pdx1-Cre* recombination of *R26R*^{EYFP}, while Cpa1+ cells appear as bright green. White boxes indicate areas with expanded views seen in the adjacent panels. (A–C) E11.5 controls contain both BrdU+ Cpa1+ distal and BrdU+ Cpa1 -proximal cells. (D–F) Distal cells from PBKO at E11.5 are BrdU labeled at levels comparable to controls (yellow arrowheads). (G–I) At E12.5, comparable BrdU labeling is seen between Cpa1+ and Cpa1- of controls. (J–L) BrdU labeling is reduced in E12.5 PBKOs, regardless of Cpa1 expression status. (M–O) Distal cells are commonly BrdU labeled in E13.5 controls. (P–R) BrdU+ cells are dramatically reduced throughout the PBKO epithelium at E13. (S) BrdU labeling indices for EYFP+ cells at E11.5, E12.5 and E13.5 reveal that maximal epithelial proliferation requires β-catenin function only after E11.5. (T) Proliferation indices calculated for the Cpa1+ population in PBKOs and controls.

new insight into the molecular mechanisms of pancreas morphogenesis and differentiation, as well as reconcile previous studies of β-catenin knockout pancreata.

Whereas several prior studies found no change in islet cell number or function following deletion of β-catenin (Murtaugh et al., 2005; Wells et al., 2007), a separate study suggested that islet cell numbers declined following β-catenin deletion, reflecting islet-restricted Wnt-β-catenin transcriptional activity (Dessimoz et al., 2005). To avoid problems of overestimating β-cell mass due to changes in the overall organ structure (Kopp et al., 2011), we used a quantitative approach that captures the absolute rather than relative volume occupied by specific pancreatic cell types, and discovered a more drastic loss of β-cells in mutant pancreata than previously reported. However, and consistent with our recent finding that postnatal β-cell proliferation is β-catenin-independent (Keefe et al., 2012), we find that islet precursor-restricted deletion of β-catenin does not recapitulate the loss of islet mass caused by deletion in MPCs. Therefore, we conclude that β-catenin acts prior to endocrine specification, maintaining and expanding MPC numbers sufficient to establish normal islet mass (Stanger et al., 2007). In the absence of β-catenin, MPCs are proximalized and prematurely generate early endocrine precursors, biased toward glucagon+ α-cells (Johansson et al., 2007), and are not subsequently available in sufficient numbers to generate the full complement of β-cells. Overall decreased islet cell genesis in the absence of β-catenin may also reflect antagonism between this protein and Notch, uncovered here, as hyperactive Notch is known to inhibit endocrine development (Hald et al., 2003; Murtaugh et al., 2003). Further studies will be required to parse the roles of β-catenin and Notch in MPCs and trunk cells.

How does β-catenin regulate MPC numbers? Importantly, we find that the initial specification, patterning and proliferation of MPCs are normal in PBKO pancreata, through E11.5, but notably aberrant as soon as 24 h later. In particular, the progressive disappearance of MPCs, and the increased number of Ngn3+ cells, indicates a defect in maintaining the early proximal–distal pattern of the organ, independent of effects on proliferation. Proliferation is impaired in PBKO pancreata, a defect that may reflect, in part, loss of expression of the Wnt target gene *cMyc* (Bonal et al., 2009; Nakhai et al., 2008). Direct inhibition of Wnt signaling in the pancreas, through expression of a dominant-negative *Fz8* mutant, impairs proliferation without any obvious effect on patterning or differentiation (Papadopoulou and Edlund, 2005). We are therefore tempted to speculate that the proliferation-specific effects of β-catenin reflect activity of the canonical Wnt signaling pathway, while effects on patterning may reflect Wnt-independent β-catenin functions, possibly including cross-talk with Notch. In future studies, we hope to address this issue with new genetic tools that we and others have developed to selectively disrupt Wnt/β-catenin signaling (Barrott et al., 2011; Valenta et al., 2011). If β-catenin has distinct modes of action, it may be possible to selectively proximalize early epithelial cells, promoting islet precursor development, while maintaining normal or even increased proliferation of progenitor cells. Such an intervention could be critical to efficiently generate β-cells from human ES or iPS cells.

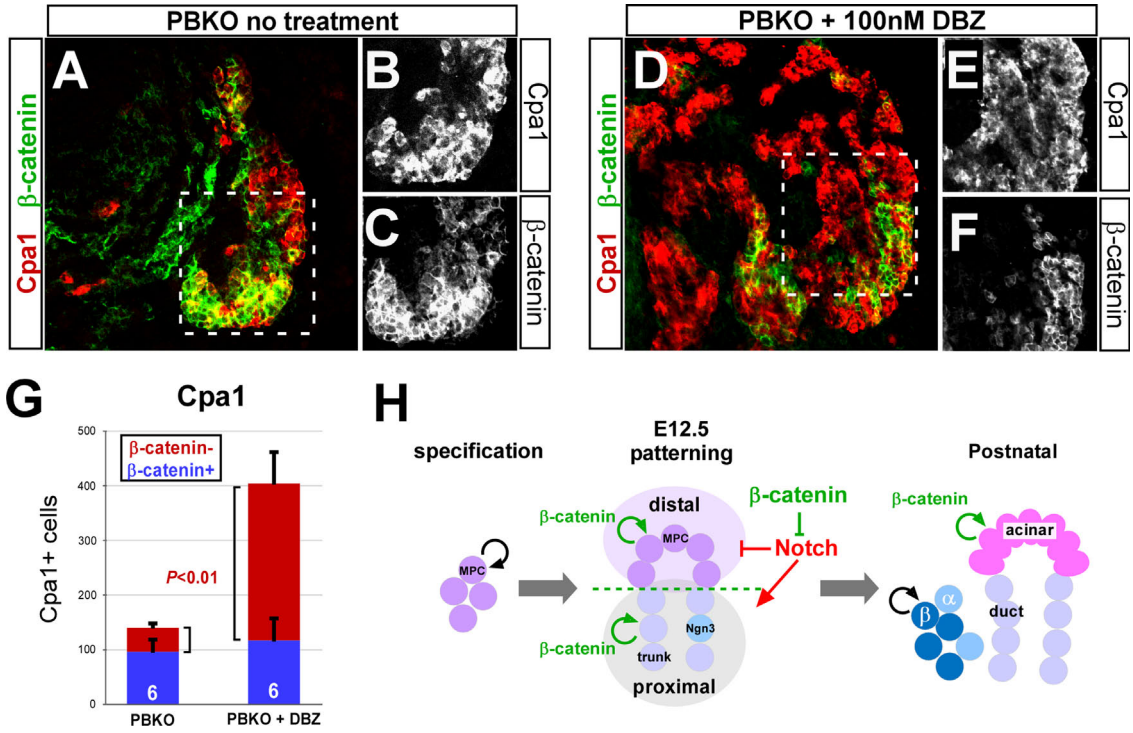

Fig. 7. Notch inhibition rescues tip/pro-acinar specification of β-catenin-deficient pancreata. PBKO and littermate control explants were cultured for 3 days with or without the γ-secretase-inhibitor DBZ (100 nM). Explants were stained by wholemount immunofluorescence with Cpa1 (red) and β-catenin (green). (A–C) Most Cpa1+ cells present in untreated PBKO explants are also β-catenin+. (D–F) The majority of Cpa1+ cells are β-catenin-deficient in DBZ treated PBKO explants. (G) Quantification of the total number of Cpa1+ cells, scored as β-catenin+ (blue portion of bar) or β-catenin- (red portion of bar), shows a significant increase in the number of Cpa1+ β-catenin- cells in DBZ treated PBKO explants. (H) We have identified a role for β-catenin in maintaining the distal pattern of early pancreatic progenitor cells, indicated by the green dashed line between distal and proximal domains at E12.5. This function appears to be mediated in part by inhibition of Notch, which induces distal-to-proximal differentiation of MPCs. Independent of its role in patterning, β-catenin is also required for maximal proliferation of tip and trunk progenitors as well as acinar cells (green circular arrows), but not for newly specified MPCs or postnatal β-cells (black circular arrows) (Keefe et al., 2012).

The fact that β-catenin is essentially dispensable before E12.5 may explain the previous paradoxical finding that early activation of β-catenin actually causes pancreas agenesis (Heiser et al., 2006). This result was obtained with *Pdx1-Cre*^{early}, which induces recombination prior to MPC specification; activation of β-catenin following the secondary transition, with *Pdx1-Cre*^{late}, results in exocrine pancreas hyperplasia. We suggest that the signaling function of β-catenin is normally inactive prior to MPC specification, and that its hyperactivation at early stages may respecify the organ to an alternative developmental fate (Heller et al., 2002; McLin et al., 2007).

The basis for the transition to β-catenin dependence, for both patterning and proliferation, remains unknown. We note, however, that it appears to occur simultaneously with several other regulatory transitions, including resolution of epithelial branching (Villasenor et al., 2010), establishment of mutually repressive Nkx6.1 and Ptf1a expression domains (Schaffer et al., 2010), and upregulation of the *Ptf1a* autoregulatory enhancer (Masui et al., 2008). Our results would be consistent with any or all of these transitions requiring β-catenin function, and we are interested to determine the mechanism or mechanisms by which β-catenin orchestrates these and other early processes to allow subsequent elaboration of organ growth and differentiation.

Acknowledgments

We thank Douglas Melton (Harvard University) for providing *Pdx1-Cre* and *Pdx1-CreERT* mice, and Chris Wright (Vanderbilt University) for providing Ptf1a antibodies. The anti-Ngn3 monoclonal antibody (I25A1B3) was obtained from the Developmental Studies Hybridoma Bank, developed under the auspices of the NICHD and maintained by The University of Iowa. We thank Matthew Keefe and Rachel Redman for helpful discussions and feedback on the manuscript. B.K.B. was a predoctoral trainee of the University of Utah Interdisciplinary Training Program in Metabolism (National Institutes of Health T32-DK091317), and this work was supported by a grant to L.C.M. from the National Institutes of Health (R01-DK075072).

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2014.03.019.

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