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Unique mechanisms of growth regulation and tumor suppression upon Apc inactivation in the pancreas

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β-catenin signaling is heavily involved in organogenesis. Here, we investigated how pancreas differentiation, growth and homeostasis are affected following inactivation of an endogenous inhibitor of β-catenin, adenomatous polyposis coli (Apc). In adult mice, Apc-deficient pancreata were enlarged, solely as a result of hyperplasia of acinar cells, which accumulated β-catenin, with the sparing of islets. Expression of a target of β-catenin, the proto-oncogene *c-myc* (*Myc*), was increased in acinar cells lacking Apc, suggesting that *c-myc* expression is essential for hyperplasia. In support of this hypothesis, we found that conditional inactivation of *c-myc* in pancreata lacking Apc completely reversed the acinar hyperplasia. Apc loss in organs such as the liver, colon and kidney, as well as experimental misexpression of *c-myc* in pancreatic acinar cells, led to tumor formation with high penetrance. Surprisingly, pancreas tumors failed to develop following conditional pancreas Apc inactivation. In Apc-deficient acini of aged mice, our studies revealed a cessation of their exaggerated proliferation and a reduced expression of *c-myc*, in spite of the persistent accumulation of β-catenin. In conclusion, our work shows that β-catenin modulation of *c-myc* is an essential regulator of acinar growth control, and unveils an unprecedented example of Apc requirement in the pancreas that is both temporally restricted and cell-specific. This provides new insights into the mechanisms of tumor pathogenesis and tumor suppression in the pancreas.

KEY WORDS: Pancreas, Growth, Apc, β-catenin, *c-myc*, ICAT, Mouse

INTRODUCTION

The size of the pancreas, a compound acinar exocrine gland also containing endocrine islets, is determined by intrinsic factors, such as the number of early progenitor cells (Stanger et al., 2007), and by extrinsic signals. The Wnt/β-catenin signaling pathway is one genetic mechanism controlling body and organ size and shape: pancreas size and the proportions of its different cell types are altered when the stability of β-catenin is modified (Heiser et al., 2006). However, the mechanism of these effects conveyed by β-catenin has not been identified.

β-catenin binds α-catenin in adherens junctions and, by regulating the transcriptional activity of T cell factors (TCFs), is a key effector of Wnt signaling (reviewed in Harris and Peifer, 2005). Wnt signaling induces a conformational change in β-catenin to favor TCF binding over that of α-catenin (Gottardi and Gumbiner, 2004a). In the absence of Wnt signaling, free cytoplasmic β-catenin is sequentially phosphorylated by a complex containing Apc and degraded. Upon Wnt binding to its receptor, on the contrary, the unphosphorylated form accumulates in the cytoplasm before being translocated into the nucleus, where it binds TCFs, thus activating the transcription of genes involved in cell proliferation (Harris and Peifer, 2005; Polakis, 1999).

Because Apc is involved in β-catenin degradation (Polakis, 1999; van Es et al., 2001), Apc inactivation creates a permissive condition whereby free unphosphorylated β-catenin may accumulate, thus mimicking active Wnt signaling (Staal et al., 2002). Here, we show that inactivating *Apc* in all pancreatic epithelial cells of early primordia induces a pancreatomegaly resulting from the selective, *c-myc*-dependent, increased proliferation of acinar cells between birth and 6 months of age. Interestingly, this very mutation in liver, colon and kidney is always tumorigenic (Andreu et al., 2005; Colnot et al., 2004; Sansom et al., 2005; Shibata et al., 1997), but not in the pancreas.

MATERIALS AND METHODS

Mice

Animals bearing exon 15 (anciently 14) of *Apc* flanked by two loxP sites ('floxed') (Shibata et al., 1997) were crossed with mice expressing Cre under the control of a *Pdx1* promoter (Herrera, 2000; Herrera et al., 2002). Tail DNAs were analyzed as described, using P3, P4 and P5 primers (Shibata et al., 1997). *c-myc*-floxed, *Pax6-Cre* and *Smad4*-floxed mice are described elsewhere (Trumpp et al., 2001; Ashery-Padan et al., 2004; Herrera et al., 2002; Yang et al., 2002). All experiments were approved by the 'Office Vétérinaire' of the State of Geneva.

Gene expression analyses

Adult pancreas RNAs were extracted as described (Glisin et al., 1974), whereas embryonic and isolated islets RNAs were extracted with the RNeasy micro kit (Qiagen). Total RNA was DNase-I-treated according to the manufacturer (Ambion). First-strand cDNA synthesis was performed using SuperScript II reverse transcriptase (Invitrogen Life Technologies). Real-time RT-PCR primers were designed with Primer express 2.0 (Applied Biosystems); three housekeeping genes were used as controls (Eef1a1, Rps9 and β-tubulin). PCRs were done in triplicate, with five specimens per condition, and were labeled with SYBR green

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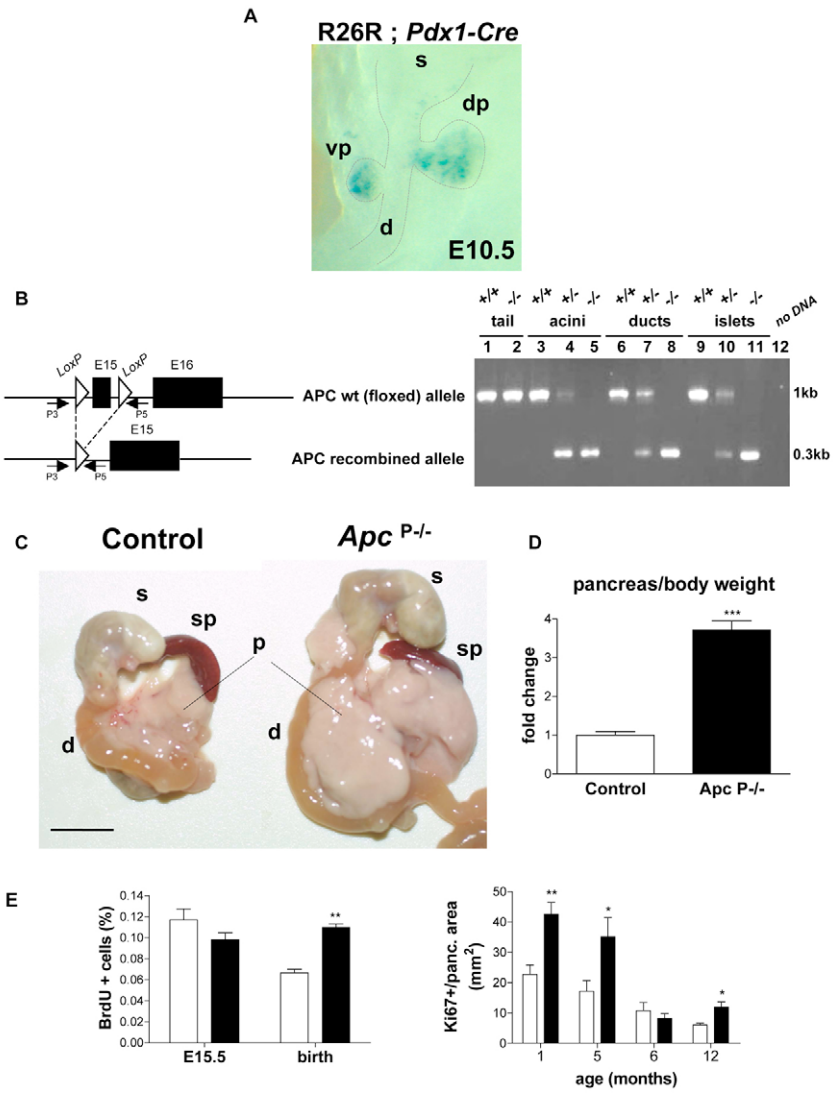

Fig. 1. Postnatal pancreatomegaly after *Apc* loss. (**A**) An E10.5 double-transgenic *R26R;Pdx1-Cre*$^{+/-}$ embryo stained with X-gal (whole mount). β-galactosidase activity is detected both in the ventral and dorsal pancreatic buds. (**B**) Genomic PCR using DNA from tail biopsies, isolated acini, ducts or islets of 2-month-old control (+/+ and +/–) and *Apc*$^{P-/-}$ mice. *Apc* is efficiently recombined in all pancreatic cell types in *Apc*$^{P-/-}$ adults (300 bp band; unrecombined tissues yield a 1 kb-long PCR fragment). (**C**) Freshly dissected pancreas of 2-month-old *Apc*$^{loxP/loxP}$ (control) and *Apc*$^{P-/-}$ mice. (**D**) Pancreas:body-weight ratio. The pancreatic mass is increased in *Apc*$^{P-/-}$ mice (pancreatic weight: control, 0.39±0.108 g; *Apc*$^{P-/-}$, 1.19±0.124 g). In total, six mice were analyzed per group; ***P<0.001. (**E**) Cell proliferation rate. The proliferation index was assayed with anti-BrdU or anti-Ki67 antibody, as indicated. White bars, controls; black bars, *Apc*$^{P-/-}$ mice (n=3 individuals per group; **P<0.01; *P<0.05). vp/dp, ventral/dorsal pancreatic primordia; s, stomach; d, duodenum; p, pancreas; sp, spleen. Scale bar: 0.5 cm in C.

master mix (Applied Biosystems). Fluorescence was quantified with the Prism 7900 HT sequence detection system (Applied Biosystems). Raw Ct (threshold cycle) values obtained with SDS 2.0 (Applied Biosystems) were used to calculate the normalization factor and the fold change with the geNorm script, as published (Vandesompele et al., 2002). No change was scored when $P \geqslant 0.05$. All experiments were performed at the Genomics Platform of our Medical School.

Immunohistochemistry

Fetal and adult pancreata were harvested in cold PBS and fixed overnight in 4% PFA (4°C). Tissues were embedded in paraffin and processed for histology. For BrdU and Ki67, slides were soaked in 10 mM citrate buffer (pH 6) in a microwave oven at 600 W for 10 minutes and were then kept for 20 minutes at room temperature (antigen retrieval). For β-catenin, slides were soaked in TEG buffer (Tris EGTA pH 9) in a microwave oven at 600 W for 5 minutes, for 15 minutes at 200 W, and for 20 minutes at room temperature. For ICAT (also known as Ctnnbip1 – Mouse Genome Informatics), slides were boiled for 10 minutes in citrate buffer (pH 6) and then left for 10 minutes at room temperature. Non-specific binding sites were blocked with 3% BSA, 0.1% Tween in PBS (30 minutes). Primary antibodies were incubated for 1 hour in blocking reagent. Antibodies used were: rabbit anti-Pdx1 (gift from C. Wright, Vanderbilt University Medical Center, Nashville, TN; 1/5000), rabbit anti-human amylase (Sigma, 1/250), guinea pig anti-porcine insulin (Dako, dilution 1/400), mouse anti-porcine glucagon (Sigma, 1/1000), rabbit anti-human somatostatin (Sigma, 1/200), rabbit anti-human PP (Bachem, 1/200), mouse monoclonal anti-BrdU (clone BU-1 from Amersham), mouse anti-human Ki67 (BD Transduction Laboratories, 1/200), mouse anti-β-catenin (BD Transduction Laboratories, 1/100), mouse anti-E-cadherin (BD Transduction Laboratories, 1/100), affinity purified rabbit anti-ICAT antibody, 1/500 (gift from C. J. Gottardi, Feinberg School of Medicine, Northwestern University, Chicago, IL). Sections were washed and incubated with specific secondary antibodies coupled either to Alexa-Fluor-488 (Molecular Probes) or Cy3 (Jackson ImmunoResearch). For ICAT immunostaining, the TSA system (Molecular Probes) was used.

Specificity of the different immunostainings was confirmed with sections in which primary antibodies were omitted. Sections were examined with a Nikon epifluorescence microscope (Eclipse TE200) equipped with a Nikon DS-L1 camera, or with a Zeiss LSM 510 confocal microscope.

Western blotting

Pancreata or islets from *Apc*$^{loxP/loxP}$, *Apc*$^{loxP/loxP}$*;Pdx1-Cre*$^{+/-}$, *Apc*$^{loxP/loxP}$*;Pax6-Cre*$^{+/-}$ and *c-myc*$^{loxP/loxP}$*;Apc*$^{loxP/loxP}$*;Pdx1-Cre*$^{+/-}$ mice were homogenized using a polytron in lysis buffer (50 mM Tris-HCl, pH 7.5, 250 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM DTT) containing complete protease inhibitors (Roche) and incubated for 30 minutes on ice. Lysates were clarified by centrifugation and protein concentration was determined. Samples were fractionated by SDS-PAGE and transferred to Immobilon P membranes (Millipore) for immunoblotting with mouse monoclonal anti-active-β-catenin 8E7 antibody (Upstate, 1/400) or with rabbit anti-β-catenin (phospho Y142;

Table 1. Expression profile (real-time PCR) of selected genes in *Apc*$^{P-/-}$ pancreata

Gene	Oligonucleotides (5′-3′)	Relative fold change (%) in *Apc*$^{P-/-}$ pancreata		
		E15.5	2 months	8-10 months
Apc	TTGGAAGTGTGAAAGCATTGATG AAGGCACTCAAAACGCTTTTG	Down 4.3× (23%)	Down 21× (5%)	Down 15× (7%)
β-catenin	CAGATCTTGGACTGGACATTGG AACGGTAGCTGGGATCATCCT	Unchanged	Unchanged	Unchanged
E-cadherin	GAGCGTGCCCCAGTATCGT GGCTGCCTTCAGGTTTTCATC	Unchanged	Unchanged	Unchanged
Tcf4	GGCGAGCAGGAGGAGAAGA GGACTTGACATCGGCTAAATCC	Unchanged	Unchanged	Unchanged
Axin2	GAAGGAAAATGAAACCAATTAAGAAGAC CCCCGCCCTCCTGAAG	Up 1.4×	Unchanged	Up 3.5×
c-jun	CTGCATGCTATCATTGGCTCAT CCACACCATCTTCTGGTGTACAG	Unchanged	Up 4.4×	Unchanged
c-myc	CCTAGTGCTGCATGAGGAGACA CCTCATCTTCTTGCTCTTCTTCAGA	Unchanged	Up 3.2×	Down 2.6× (38%)
Cdk4	CCCACCTCTCCTTACGAGGTT AGAAGACAGATACACCTGCCCTTTA	Unchanged	Up 2.3×	Unchanged
p21	GCAGACCAGCCTGACAGATTTC GGCACTTCAGGGTTTTCTCTTG	Unchanged	Unchanged	Unchanged
cyclin D2	CAGAAGGACATCCAACCGTACAT CACTTTTGTTCCTCACAGACCTCTAG	Up 1.5×	Up 2.8×	Unchanged

For each gene, forward primers are shown in the first sequence and reverse primers in the second. Expression levels are relative to transcripts in control (*Apc*$^{loxP/loxP}$) pancreata, which were calculated using three different housekeeping genes (Materials and methods), and normalized to 1 (100%). *Apc* transcripts were measured using primers placed at the junction between exon 14 and 15 (floxed). Five animals were used per condition (three at 8-10 months). Real-time RT-PCR reactions were performed in triplicate. $P<0.01$.

Abcam, 1/500) and with rabbit polyclonal anti-β-tubulin antibody (Abcam ab6046, 1/2000). Detection was performed using peroxidase-conjugated anti-mouse or anti-rabbit IgGs (Promega, 1/5000). Bands were visualized by chemiluminescence (ECL, Amersham) according to the manufacturer's instructions.

BrdU treatment

Animals were given BrdU (Sigma) intraperitoneally (50 μg/g of body weight) 2 hours prior to sacrifice.

Glucose tolerance

Animals were fasted overnight (16 hours) and injected intraperitoneally with 2 g glucose (Fluka) per kg of body weight. Glycemia was measured using Glucometer DEX (Bayer) strips. Insulinemia was determined by ELISA (Kit Mercodia Ultrasensitive Rat Insulin ELISA).

Pancreatic glucagon and insulin content

Pancreatic protein extracts were prepared by adding acid-ethanol solution (74% ethanol, 1.4% HCl) and then performing homogenization. Samples were sonicated and centrifuged, and the supernatant was used for radioimmunoassay experiments performed following the manufacturer's instructions (Glucagon RIA Kit, Linco, for glucagon; and ELISA Kit Mercodia Ultrasensitive Rat Insulin ELISA for insulin).

Islet isolation

Islets from 1-month-old A*pc*$^{loxP/loxP}$, *Apc*$^{loxP/loxP}$*;Pdx1-Cre*$^{+/-}$ and *Apc*$^{loxP/loxP}$*;Pax6-Cre*$^{+/-}$ mice were isolated as described above (with collagenase type V Sigma #C-9263) and purified on a Ficoll gradient (Sigma Histopaque #1077) (Wollheim et al., 1990).

Amylase activity

Adult mouse pancreata were homogenized in 3 ml of PBS and centrifuged for 1 minute at 70.86 ***g***. Blood samples from the retro orbital sinus were collected into lithium-heparin-treated vials and centrifuged for 5 minutes at 784 ***g***. Amylase activity was assessed by enzymatic photometry using α-amylase CC FS (y; DiaSys) relative to the protein content determined by the Bradford test.

Morphometry

Three 8-week-old animals per group were analyzed. Paraffin sections obtained at 200-μm intervals were immunostained with anti-insulin antibody. Photographs were obtained with an EOS D30 digital camera (Canon) and analyzed with the National Institutes of Health (NIH) ImageJ 1.60 software. β-cell area was expressed as a percentage of total pancreatic area.

Statistics

All results were reported as mean±s.e.m. (standard error of the mean). Groups were compared with independent *t*-tests (unpaired and two-tailed), reported as *P* values. All tests were performed using the GraphPad Prism software.

RESULTS AND DISCUSSION

Apc loss leads to postnatal pancreatomegaly due to acinar hyperplasia

In order to investigate the role of β-catenin signaling in pancreas organogenesis and homeostasis, we took advantage of an existing mouse line in which exon 15 of the two *Apc* alleles was flanked by loxP sites [designated *Apc*$^{loxP/loxP}$ or 'control' mice (Shibata et al., 1997)]. These mice were bred to *Pdx1-Cre*$^{+/-}$ transgenics (Herrera, 2000; Herrera et al., 2002), which express Cre recombinase in all pancreatic epithelial cell types from embryonic day (E)10.5 (Fig. 1A). In *Apc*$^{loxP/loxP}$*;Pdx1-Cre*$^{+/-}$ mice (*Apc*$^{P-/-}$ hereafter), *Apc* was selectively invalidated in acinar, ductal and islet cells (Fig. 1B).

Control and heterozygous (*Apc*$^{loxP/+}$*;Pdx1-Cre*$^{+/-}$) mice showed undistinguishable phenotypes. *Apc*$^{P-/-}$ animals appeared normal throughout embryogenesis and at birth but, from 3 weeks of age, a marked pancreatomegaly ensued, with a pancreas-to-body weight ratio three- to five-fold higher than in control mice (Fig. 1C,D). The increased size of the pancreas persisted during the whole period of study, and is consistent with previous observations involving the forced expression of β-catenin in pancreata (Heiser et al., 2006).

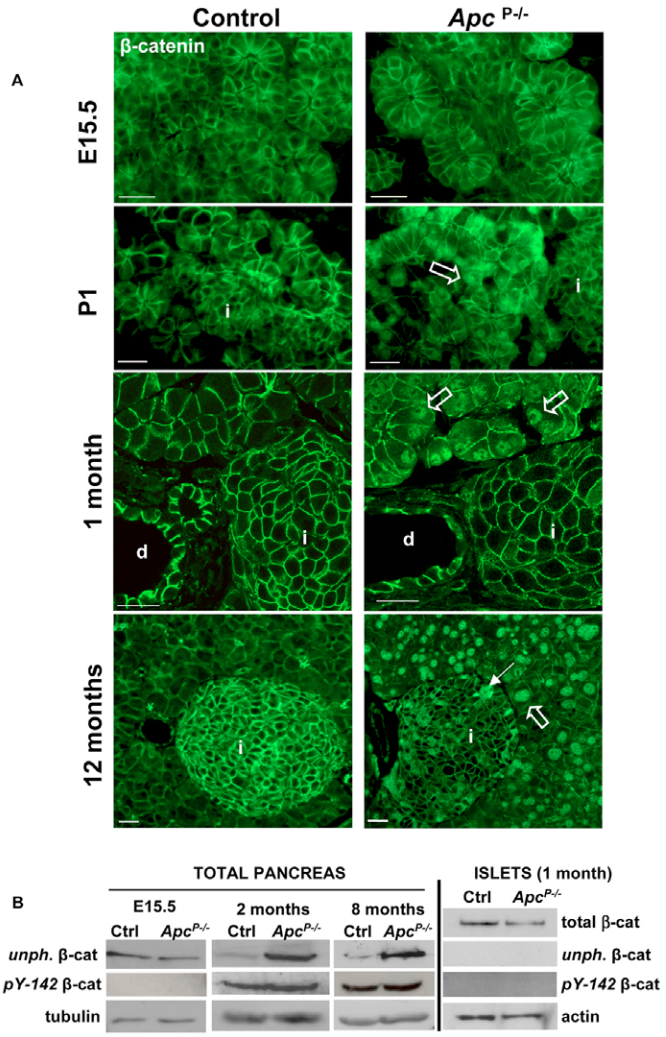

Fig. 2. β-catenin accumulates in *Apc*$^{P-/-}$ acinar cells. (**A**) Anti-pan β-catenin immunofluorescence at E15.5, birth (P1), 1 month (confocal pictures are shown for this stage) and 12 months of age. In control mice (left column), β-catenin staining is found in cell membranes at all stages analyzed. By contrast, in adult *Apc*$^{P-/-}$ mice (right column) staining for β-catenin is mainly nuclear in acinar cells from P1 (open arrows) but remains peripheral in most islet and ductal cells, as in controls. Notice that some islet cells display a cytoplasmic β-catenin staining (small arrow) in old *Apc*$^{P-/-}$ mice. (**B**) Western blot of total protein extracts from E15.5 and adult pancreata (50 μg per lane) using anti-unphosphorylated β-catenin antibody and anti-β-catenin-(phospho Y142) antibody. Unphosphorylated β-catenin accumulates only in pancreatic extracts from *Apc*$^{P-/-}$ adult mice and is always undetectable in isolated islets (right). β-catenin-(phospho Y142) is abundant in adult acini, but undetectable in developing primordia or in isolated islets (1-month-old), whether control or *Apc*$^{P-/-}$. β-tubulin and actin (lower row) show equal loading. d, duct; i, islet. Scale bars: 20 μm.

Acinar, ductal and islet cells appeared histologically normal in *Apc*$^{P-/-}$ fetuses and young adults (1-4 months old; see Fig. S1 in the supplementary material), yet the islets of Langerhans appeared diluted (i.e. more scarce within a hyperplastic exocrine compartment). Cell density was comparable in control and *Apc*$^{P-/-}$ young adults (data not shown), indicating that pancreatomegaly was due to acinar cell hyperplasia, rather than to acinar cell enlargement (hypertrophy). Cell proliferation and differentiation were normal in fetal pancreata but, from birth until 6 months of age, acinar cells had an increased proliferation rate (Fig. 1E); the rates of cell proliferation were normal in ducts and islets (data not shown).

Exocrine and endocrine functions of *Apc*$^{P-/-}$ pancreata were normal. Absolute pancreatic amylase activity was normal despite acinar hyperplasia (see Fig. S2A,B in the supplementary material), suggesting a decreased amylase content per cell. This could result from adaptation to an excessive acinar cell mass or from defective acinar cell differentiation. Of particular interest, compared with controls, *Apc*$^{P-/-}$ mice were hyperamylasemic (see Fig. S2C in the supplementary material), suggesting that plasma amylase might be a good indicator of the functional pancreatic mass.

Although adult islet density was reduced, the absolute β-cell mass in *Apc*$^{P-/-}$ animals was unaffected, as were total pancreatic insulin and glucagon contents, and glucose homeostasis (see supplementary Fig. S1B and Fig. S3 in the supplementary material).

Altogether, these observations indicate that only acinar cells are sensitive to Apc loss and that the endocrine-to-exocrine tissue ratio [1-99% (Orci, 1982)] is not crucial in long-term pancreas homeostasis.

Sensitivity of acinar cells to β-catenin signaling is restricted to a postnatal competence period

Apc inactivation is expected to induce the accumulation of unphosphorylated β-catenin (Staal et al., 2002). Surprisingly, despite the efficient inactivation of Apc in *Apc*$^{P-/-}$ fetuses (E15.5), the expression and distribution of β-catenin was not changed compared to controls (Fig. 2A,B); accordingly, expression of β-catenin target genes was not, or only slightly, upregulated (Table 1). By contrast, in the postnatal pancreas, Apc loss elicited the nuclear accumulation of β-catenin in acinar cells from birth (Fig. 2A). This unexpected difference confirms the compartmentalization of the effects of Apc loss to postnatal acinar lineages.

To analyze the basis of acinar cell hyperplasia in *Apc*$^{P-/-}$ mice, we examined the expression profile of different β-catenin target genes, such as *c-myc*, *c-jun* (*Jun*) and cyclin D2 (*Ccnd2*) (Table 1) (Hurlstone and Clevers, 2002). These transcripts were markedly increased in 2-month-old *Apc*$^{P-/-}$ total pancreatic extracts; by contrast, this was not the case in islets isolated from 1-month-old *Apc*$^{P-/-}$ animals (data not shown).

In young adult *Apc*$^{P-/-}$ mice, and more markedly in aged animals, β-catenin accumulation was apparent in a fraction of ductal and islet cells, and was diffuse, both cytoplasmic and nuclear (Fig. 2A,B). The refractoriness of islet cells to β-catenin signaling was further established with mice bearing the *Apc* inactivation exclusively in islets through the use of a *Pax6-Cre* transgene (Ashery-Padan et al., 2004; Herrera et al., 2002) (*Apc*$^{loxP/loxP}$;*Pax6-Cre*$^{+/-}$). In these mutants, no islet dysplasia or hyperplasia was observed (see Fig. S4A in the supplementary material). Despite the efficient downregulation of *Apc* transcripts (80%) in *Apc*$^{loxP/loxP}$;*Pax6-Cre*$^{+/-}$ islets, the expression of β-catenin target genes, such as in *Apc*$^{P-/-}$ islets, was not augmented (see Fig. S4B in the supplementary material).

In conclusion, these results reveal a precise spatiotemporal pattern in the pancreas for β-catenin signaling: it remains low or inactive during pancreas development but, after birth, is activated in acinar cells only.

Interestingly, β-catenin signaling (i.e. 'activation' of β-catenin, or its nuclear translocation) can occur in the absence of Wnt signaling via other mechanisms (Harris and Peifer, 2005; Willert and Jones,

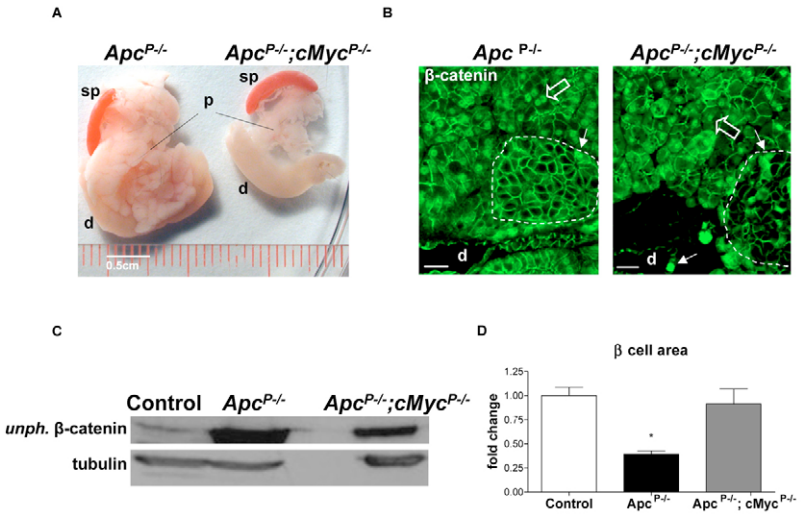

Fig. 3. Pancreatomegaly is *c-myc* dependent. (**A**) Freshly dissected pancreata from 2-month-old $Apc^{P-/-}$ and $Apc^{P-/-}$;*c-myc*$^{P-/-}$ mice. Pancreatomegaly does not appear in the absence of the two *c-myc* alleles. (**B**) Anti-β-catenin immunofluorescence on 2-month-old $Apc^{P-/-}$ and $Apc^{P-/-}$;*c-myc*$^{P-/-}$ pancreatic sections. Notice the nuclear localization of β-catenin in acinar cells of mice of both genotypes (open arrows). Islets are depicted with a dashed line. Paraffin sections were 5 μm thick. Small arrows show cytoplasmic β-catenin in islets and ducts (d). (**C**) Western blot of total protein extracts from adult pancreas (50 μg per lane) using anti-unphosphorylated β-catenin antibody. Unphosphorylated β-catenin accumulates in the pancreas of $Apc^{P-/-}$;*c-myc*$^{P-/-}$ mice, as in $Apc^{P-/-}$ animals. β-tubulin shows equal loading. (**D**) β-cell area in $Apc^{P-/-}$;*c-myc*$^{P-/-}$ mice is similar to that of controls, whereas it is lower in $Apc^{P-/-}$ animals. A total of three mice were analyzed per group; *P<0.01. d, duodenum (A), duct (B); p, pancreas; sp, spleen. Scale bar: 0.5 cm in A; 20 μm in B.

2006). β-catenin-(phospho Y142) is an indicator of Wnt-independent β-catenin signals (Brembeck et al., 2004). Phosphorylation of β-catenin at tyrosine 142, mediated by hepatocyte growth factor (Hgf) receptors (Met), has been shown to occur in murine hepatomegaly (Apte et al., 2006), human hepatoblastomas (Ranganathan et al., 2005) and human colorectal carcinoma cells (Rasola et al., 2006). In the pancreas, we found that β-catenin-(phospho Y142) levels were undetectable in pancreatic primordia and in isolated islets, but high in acinar cells, whether control or $Apc^{P-/-}$ (Fig. 2B). This expression pattern correlates with that of unphosphorylated nuclear β-catenin in $Apc^{P-/-}$ pancreata.

Taken together, these observations suggest that Wnt-dependent and -independent β-catenin signaling are intrinsically facilitated in adult acinar cells.

Deletion of *c-myc* is sufficient to abolish the $Apc^{P-/-}$ phenotype

To test whether the increased *c-myc* expression observed in $Apc^{P-/-}$ mutants is required for the acinar overexpansion, we performed the double inactivation of *Apc* and *c-myc* using mice bearing, in addition to the two loxP-flanked *Apc* alleles, two loxP-flanked *c-myc* alleles (Trumpp et al., 2001). Remarkably, mice simultaneously lacking *Apc* and *c-myc* ($Apc^{P-/-}$;*c-myc*$^{P-/-}$) in pancreas displayed a complete reversal of the $Apc^{P-/-}$ phenotype, with no pancreatomegaly in spite of the accumulation of nuclear β-catenin in acinar cells (Fig. 3A-C). These mice showed normal relative β-cell area (Fig. 3D) and pancreatic insulin content at 2 months of age.

Together, these results indicate that the proliferative effect of β-catenin on acinar cells after Apc loss requires increased *c-myc* activity. This is the first evidence, together with two reports on *Apc* inactivation in the intestine (Ignatenko et al., 2006; Sansom et al., 2007), which appeared while this work was under evaluation for publication, for an in vivo molecular mechanism (mediated by *c-myc* inactivation) involved in the reversal of a 'Wnt gain-of-function' (i.e. Apc deficiency) phenotype.

$Apc^{P-/-}$ pancreata become 'resistant' to β-catenin signaling and escape tumorigenesis

Mice bearing the same *Apc* mutation in liver, colon or kidney (Andreu et al., 2005; Colnot et al., 2004; Sansom et al., 2005; Shibata et al., 1997) always develop tumors. Similarly, continued overexpression of *c-myc* in acinar cells under the control of an elastase promoter is tumorigenic (Sandgren et al., 1991), and human pancreatic cancer cells have high levels of *c-myc* expression (Buchholz et al., 2006). However, contrary to these observations, tumor formation was prevented in $Apc^{P-/-}$ pancreata, despite the high levels of β-catenin.

In $Apc^{P-/-}$ animals, pancreatomegaly remained stable and, up to 1 year of age, mice were in good health, had unchanged pancreatic mass and normal endocrine function (see Fig. S3D in the supplementary material). In 1-year-old mice, hypertrophic acinar cells, with dysplastic nuclei, were observed focally (Fig. 2A; and see below and Fig. S5 in the supplementary material). However, E-cadherin expression was always normal (data not shown) and no tumors developed.

The absence of pancreatic tumors was further explored in mice lacking, simultaneously, Apc and Smad4 in the pancreas. Smad4, a central transducer of signals conveyed by Tgfβ ligands, is often mutated or deleted in colorectal cancer and pancreatic carcinoma (Bardeesy et al., 2006; Hahn et al., 1996; Hua et al., 2003; Shattuck-Brandt and Dubois, 1999; Tang et al., 2002). However, mice lacking *Smad4* in the pancreas have no pancreatic tumors (Simeone et al., 2006). In the present study, we analyzed the cumulative effect of the concurrent loss of both *Apc* and *Smad4*, and no spontaneous pancreatic tumor developed during the first year of life.

Whereas nuclear β-catenin in acinar cells persisted in 1-year-old mice (Fig. 2A), the expression of *c-myc* and other genes that are upregulated in young pancreata returned to normal levels in mature animals (Table 1), indicating the acquisition of 'resistance' to signaling via β-catenin. This correlates with the normalization of acinar cell proliferation after 5 months of age in $Apc^{P-/-}$ animals, and the absence of tumorigenesis (Fig. 1E and see Fig. S6 in the supplementary material).

The first months of life thus represent a competence window, a sensitive period during which acinar cells may undergo β-catenin-induced proliferation. The spontaneous downregulation of β-catenin signaling in the exocrine pancreas in aged mice, despite the persistent presence of abundant nuclear β-catenin, defines the beginning of a β-catenin-unresponsive phase (summarized in Fig. S6 in the supplementary material). Our observations indeed suggest a mechanism of tumor suppression, or rather of signal adaptation, after Apc loss: a resetting of the threshold upon continuous signaling by β-catenin. A similar negative-feedback mechanism after

inactivation of another tumor suppressor gene, *NF1* (whose malfunction underlies the familial cancer syndrome neurofibromatosis type I), was recently reported (Courtois-Cox et al., 2006).

The refractoriness of islet cells to convey growth signals through β-catenin might also help understand why insulinomas, which very often display a loss of APC protein expression (Arnold et al., 2007), are largely benign tumors (Gonzalez-Gonzalez and Recio-Cordova, 2006), and why β-cells do not transdifferentiate into ductal cells in pancreatic metaplastic lesions (Strobel et al., 2007).

The blockade of β-catenin/TCF activity, revealed by the persistence of nuclear β-catenin in acini without increased *c-myc* expression or cell proliferation, possibly results from the activity of β-catenin competitors [i.e. ICAT (also known as Ctnnbip1 – Mouse Genome Informatics) and Duplin (also known as Chd8 – Mouse Genome Informatics)], TCF repressors [Groucho (Tle1), Hbp1, CtBP1], or from TCF post-translational modifications (Kikuchi et al., 2006). Among the negative modulators of β-catenin that we analyzed, we found that ICAT (inhibitor of β-catenin and Tcf4), which prevents the binding of β-catenin to TCF (Gottardi and Gumbiner, 2004b; Tago et al., 2000), has a pancreatic expression pattern consistent with the observed spatial and temporal oscillations of *c-myc* expression: islet cells maintain high levels of ICAT throughout life; in acinar cells, ICAT is downregulated in mature mice, but not in $Apc^{P-/-}$ animals (see Fig. S5 in the supplementary material). Whether ICAT or other repressors are involved in this β-catenin blockade will be addressed in further studies.

In conclusion, using an in vivo system in which endogenous β-catenin signaling is allowed or favored (i.e. the ablation of Apc), we report that early pancreatic progenitor cells, and islet cells, exhibit strong inhibition of excessive β-catenin signaling (see Fig. S6B in the supplementary material). Others have shown that forced excessive or defective β-catenin signaling disrupts normal acinar organogenesis (Dessimoz et al., 2005; Heiser et al., 2006; Murtaugh et al., 2005; Papadopoulou and Edlund, 2005; Wells et al., 2007). Later in life, upregulation of β-catenin inhibitors/competitors, leading to impaired β-catenin/TCF activity, would block signaling in acini (see supplementary material Fig. S6B).

Whether β-catenin signaling does play a role during pancreas development, growth and maturation remains to be clearly determined. Here, we have demonstrated that, at least in a favorable situation for β-catenin signaling (such as after Apc loss), the definitive size of the pancreas at complete maturation appears to be intrinsically determined by the progressive unresponsiveness to β-catenin signaling in acinar cells; in addition, this endogenous constraint probably explains why tumor formation is prevented in pancreas but not in liver, colon or kidney bearing the same *Apc* mutation (Andreu et al., 2005; Colnot et al., 2004; Sansom et al., 2005; Shibata et al., 1997), and might be of therapeutic relevance.

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Supplementary material

Supplementary material for this article is available at
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