

Rb and p53 Execute Distinct Roles in the Development of Pancreatic Neuroendocrine Tumors



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ABSTRACT

Pancreatic neuroendocrine tumors (PanNET) were classified into grades (G) 1 to 3 by the World Health Organization in 2017, but the precise mechanisms of PanNET initiation and progression have remained unclear. In this study, we used a genetically engineered mouse model to investigate the mechanisms of PanNET formation. Although pancreas-specific deletion of the *Rb* gene (*Pdx1-Cre;Rb^{fl/fl}*) in mice did not affect pancreatic exocrine cells, the α -cell/ β -cell ratio of islet cells was decreased at 8 months of age. During long-term observation (18–20 months), mice formed well-differentiated PanNET with a Ki67-labeling index of 2.7%. In contrast, pancreas-specific induction of a *p53* mutation (*Pdx1-Cre;Trp53^{R172H}*) had no effect on pancreatic exocrine and endocrine tissues, but simultaneous induction of a *p53* mutation with *Rb* gene deletion (*Pdx1-Cre;Trp53^{R172H};Rb^{fl/fl}*) resulted in the formation of aggressive PanNET

with a Ki67-labeling index of 24.7% over the short-term (4 months). In *Pdx1-Cre;Trp53^{R172H};Rb^{fl/fl}* mice, mRNA expression of *Pten* and *Tsc2*, negative regulators of the mTOR pathway, significantly decreased in the islet cells, and activation of the mTOR pathway was confirmed in subsequently formed PanNET. Thus, by manipulating *Rb* and *p53* genes, we established a multistep progression model from dysplastic islet to indolent PanNET and aggressive metastatic PanNET in mice. These observations suggest that *Rb* and *p53* have distinct roles in the development of PanNET.

Significance: Pancreas-specific manipulation of *Rb* and *p53* genes induced malignant transformation of islet cells, reproducing stepwise progression from microadenomas to indolent (grade 1) and subsequent aggressive PanNETs (grade 2–3).

Introduction

Pancreatic neuroendocrine neoplasms (PanNEN) are the second most common epithelial neoplasms in the pancreas, and the number of people affected is gradually increasing (1). In 2017, the World Health Organization (WHO) classified human PanNENs into two groups: well-differentiated PanNENs, called PanNETs, and poorly differentiated PanNENs, called PanNECs. PanNECs, which include small and large cell carcinomas, have an extremely poor prognosis and are thought to be biologically distinct from pancreatic neuroendocrine tumors (PanNET; refs. 2–5). PanNETs are further subclassified into

grades (G) 1 to 3 on the basis of their proliferative activity assessed by the Ki67 labeling index and mitotic rate. Although the prognosis of patients with PanNETs correlates with the tumor grade, the underlying mechanisms of PanNET formation and grade progression are unclear.

Previous gene analyses of PanNETs revealed major mutations in *MEN1*, *DAXX*, *ATRX*, and genes in the mTOR pathway, but extremely rare mutations in the retinoblastoma (*RB*) gene or *TP53* gene (6–8). In contrast, a recent report analyzed by IHC revealed a loss of *Rb* expression in 54.5% of G3 PanNEC but not in G3 PanNET cases (5). However, in various human neoplasms, the *RB* gene is functionally inactivated not only by mutations, but also by altered expression of upstream regulators (9). Indeed, a recent study demonstrated that increased expression of *CDK4/6* via copy number abnormalities leads to *Rb* phosphorylation in 46% to 68% of human PanNETs, resulting in inactivation of the *Rb* pathway (10, 11). Likewise, aberrant activation of MDM2, MDM4, and WIP1 suppresses the *p53* signaling pathway in approximately 70% of PanNETs (12). The involvement of *Rb* and *p53* in the tumorigenic process in PanNETs is further suggested by findings from genetically engineered mouse models. For example, the *RIP1-Tag2* mouse, in which transgenic expression of the simian virus 40 (SV40) large T antigen is under control of the *rat insulin promoter* (*RIP*), develops aggressive insulinomas through suppression of both the *Rb* and *p53* pathways (13). Similarly, *preproglucagon promoter*-driven expression of SV40 large T antigen results in moderate to aggressive glucagonomas (14, 15), and homozygous deletion of *Rb* and *p53* in renin-expressing cells leads to the development of aggressive glucagonomas in the pancreas (16). These models highlight the importance of simultaneous inactivation of both the *Rb* and *p53* pathways in the development of aggressive PanNETs in mice. Whether these aggressive tumors develop from indolent tumors, however, remains unknown.

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Furthermore, no studies have investigated the individual roles of Rb and p53 in PanNET formation from pancreatic cells.

In this study, we investigated the roles of Rb and p53 in the development of PanNETs utilizing a genetically engineered mouse model. We provide the first reported evidence that *pancreas duodenum homeobox protein 1 (Pdx1)* Cre-dependent pancreas-specific deletion of *Rb* gene *per se* induces indolent PanNETs in islet cells. Although pancreas-specific induction of a *p53* mutation alone had no effect on pancreatic tissue, it markedly accelerated the progression of PanNETs in combination with *Rb* deletion. These data suggest that Rb and p53 have distinctive roles in the development of PanNETs.

Materials and Methods

Mice

We used *Pdx1-Cre* mice (17), *Rosa26R* mice (18), *Rb flox* mice (19), and *LSL-Trp53^{R172H}* mice (20, 21), which were previously described. Nonrecombinant littermates were used as controls. All mice were maintained in a specific-pathogen-free facility at the Kyoto University Faculty of Medicine (Kyoto, Japan). All experiments were approved by the institutional animal ethics committee and performed according to the guidelines of the animal ethics committee of Kyoto University.

Histologic study

Mouse tissues were fixed with 10% formalin for 24 hours, embedded in paraffin, and dissected in 5- μ m sections, which were stained with hematoxylin and eosin for histologic analysis. For IHC analysis, additional sections were deparaffinized, rehydrated with ethanol, and treated with 0.3% hydrogen peroxide and methanol for 15 minutes to inhibit endogenous peroxidase activity. Heat-mediated antigen retrieval was performed with 10 mmol/L citrate buffer (pH 6.0), and samples were incubated in a serum-free protein block (X0909; DAKO). Sections were incubated with primary antibody overnight at 4°C and with biotinylated secondary antibody for 1 hour at room temperature. The specimens were then incubated with avidin–biotin–peroxidase complex (Vectastain ABC Kit, Vector Laboratories) at room temperature for 30 minutes, stained with diaminobenzidine substrate (DAKO), and counterstained with hematoxylin. For immunofluorescence analysis, sections were incubated with primary antibody overnight at 4°C and incubated with fluorophore-conjugated secondary antibody (Invitrogen) for 1 hour at room temperature. Primary antibodies used in this study were mouse anti-cytokeratin (1:100; clone AE1/AE3, M3515; DAKO), rabbit anti-pancreatic alpha amylase (1:300, ab21156; Abcam), rabbit anti-chromogranin A (1:1000; ab15160; Abcam), mouse anti-synaptophysin (1:200; M7351; DAKO), guinea pig anti-insulin (1:400; A0564; DAKO), rabbit anti-glucagon (1:300; A0565; DAKO), rabbit anti-somatostatin (1:500; ab22682; Abcam), goat anti-pancreatic polypeptide (PP; 1:400; ab77192; Abcam), rat anti-Ki67 (1:300; M7249; DAKO), rabbit anti-p53 (1:500; clone CM5, VP-P956; Vector Laboratories), and rabbit anti-phospho-S6 ribosomal protein (1:400; Ser235/236, #2211S; Cell Signaling Technology). Phospho-S6 ribosomal protein was scored by applying a semiquantitative immunoreactivity method (*H*-score) as described previously (22).

β -Galactosidase (LacZ) staining

Mice were anesthetized and briefly perfused intracardially with ice-cold fixative solution (PBS containing 4% paraformaldehyde, 30% sucrose, 0.5 mmol/L EGTA, 2 mmol/L MgCl₂, and 1% glutaraldehyde). Fixed pancreas tissues were mounted and dissected in 8- μ m sections. β -Galactosidase substrate [5 mmol/L K₃Fe(CN)₆, 5 mmol/L K₄Fe

(CN)₆, and 1 mg/mL X-galactosidase in rinse buffer (PBS containing 2 mmol/L MgCl₂, 0.02% NP-40, and 0.1% sodium deoxycholate)] was added to the sections, which were then incubated in the dark overnight at room temperature. The sections were washed twice with rinse buffer for 5 minutes, fixed with 10% formalin for 5 minutes, and counterstained with nuclear fast red (KPL).

Islet isolation and RNA isolation

Islets of Langerhans were isolated from *Pdx1-Cre*, *Pdx1-Cre;Rb^{f/f}*, and *Pdx1-Cre;Trp53^{R172H};Rb^{f/f}* mice at 4 to 8 months of age using the collagenase digestion technique (23). Briefly, Hank's Balanced Salt Solution (HBSS) containing 0.5 mg/mL collagenase P (#11213865001; Roche) was injected into the mouse pancreas via the bile duct. The pancreas was subsequently removed and further digested in HBSS containing 0.5 mg/mL collagenase P for 30 minutes at 37°C. The digested pancreas was washed twice with ice-cold Krebs–Ringer bicarbonate (KRB) buffer (129.4 mmol/L NaCl, 5.2 mmol/L KCl, 2.7 mmol/L CaCl₂, 1.3 mmol/L KH₂PO₄, 1.3 mmol/L MgSO₄, and 24.8 mmol/L NaHCO₃ (equilibrated with 5% CO₂/95% O₂, pH 7.4)] containing 2.8 mmol/L glucose, suspended in 4 mL of histopaque 1.119 (#11191; Sigma), and transferred to a clean glass tube; 2 mL of histopaque 1.077 (#10771; Sigma) and 2 mL of histopaque 1.050 (prepared by mixing two volumes of histopaque 1.077 with one volume of distilled water) were then sequentially overlaid to perform a density gradient separation. After 800 $\times$ *g* centrifugation for 10 minutes at room temperature, the islets observed in the interphase between histopaque 1.050 and histopaque 1.077 were collected and washed twice in ice-cold KRB buffer. The resulting islets were transferred to a large dish filled with approximately 50 mL of ice-cold KRB buffer and hand-picked into a 1.5-mL tube before total RNA preparation. Total RNA was prepared from the isolated islets with an RNeasy Mini Kit according to the manufacturer's instructions (#74104; Qiagen), and the RNA concentration was measured using a Qubit RNA HS Assay Kit (#Q32855; Thermo Fisher Scientific).

Quantitative real-time PCR

Single-stranded cDNA was prepared with Superscript III (Invitrogen), and qRT-PCR was performed with a LightCycler FastStart DNA Master SYBR Green 1 Kit (Roche Diagnostic). Values are expressed as arbitrary units relative to the expression of GAPDH. Primers for quantitative PCR are as follows: *Rb* gene, *Rb*-forward (5'-TACACTCTGTGACGCGCTTC) and *Rb*-reverse (5'-TTCACCTTGCAGATGCCATA); *Pten* gene, *Pten*-forward (5'-TGCACAGTATCCTTTGAAGACC) and *Pten*-reverse (5'-GAATTGCTGCAACATGATTGTCA); and *Tsc2* gene, *Tsc2*-forward (5'-GAGCCCCAAACAAGGCGCTGA) and *Tsc2*-reverse (5'-AGGCTGGCGCTCGTAAGGGAT).

Gene expression microarray analysis

The quality of RNA extracted from the isolated islets of *Pdx1-Cre* and *Pdx1-Cre;Rb^{f/f}* mice was examined with an Agilent 2100 Bioanalyzer (Agilent Technologies). The RNA samples were labeled with a Sure Tag Complete DNA Labeling Kit (Agilent Technologies) and hybridized to the SurePrint G3 Mouse GE 8 $\times$ 60K Microarray Kit (Agilent Technologies) with a Gene Expression Hybridization Kit (Agilent Technologies). The raw data were quantified in Agilent Feature Extraction software (Agilent Technologies). Quantified data were normalized by Gene Spring 12.5 software (Agilent Technologies). The pathway analysis of the gene expression data was performed with DAVID 6.8. Full microarray data have been uploaded to the Gene Expression Omnibus (GEO) under accession number GSE152582.

Measurement of serum insulin and blood glucose levels

For measurement of serum insulin and blood glucose levels, the mice underwent a 4-hour fast before collection of whole blood via orbital bleeding or a tail cut. Blood glucose was measured with a glucose meter (Glutest Every; Sanwa Kagaku Kenkyusho Co.). Serum was collected through centrifugation, snap-frozen, and stored at -80°C . Serum insulin levels were measured with a Mouse Insulin ELISA Kit (Mercodia) according to the manufacturer's instructions.

Statistical analysis

Data are presented as mean $\pm$ SE, and were analyzed with two-tailed independent-sample Student *t* test, as appropriate. *P* values < 0.05 were considered statistically significant (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

Results

Pancreas-specific *Rb* gene deletion does not affect exocrine cells, but decreases the α -cell/ β -cell ratio in islet cells

To investigate the role of the *Rb* gene in the development of pancreatic tissue, we generated pancreas-specific *Rb*-deleted mice by crossing *Pdx1-Cre* mice with *Rb* flox mice (*Pdx1-Cre;Rb^{f/f}*; Fig. 1A). After confirming in *Pdx1-Cre;Rosa26R* mice that the *Pdx1* promoter induces Cre recombinase expression in the acinar, duct, and islet cells, as reported previously (Supplementary Fig. S1A), we analyzed the *Pdx1-Cre;Rb^{f/f}* mice at 2, 4, 6, and 8 months of age. Histopathologic analysis revealed normal exocrine glandular components in both the *Pdx1-Cre;Rb^{f/f}* mice and *Pdx1-Cre* control mice (Supplementary Fig. S1B). IHC analysis revealed normal amylase staining and pan-cytokeratin staining in the acinar cells and duct cells, respectively, in *Pdx1-Cre;Rb^{f/f}* mice (Supplementary Fig. S1B). The number of Ki67-positive acinar cells and duct cells did not differ significantly between *Pdx1-Cre;Rb^{f/f}* mice and *Pdx1-Cre* control mice (Supplementary Fig. S1C and S1D). These findings suggested that pancreas-specific *Rb*-deletion has no effect on the development of exocrine cells.

In islet cells in *Pdx1-Cre;Rb^{f/f}* mice, there were no morphologic abnormalities, but the ratio of α - to β -cells was significantly lower than that in *Pdx1-Cre* control mice (Fig. 1B and C). The lower α -cell/ β -cell ratio appeared to be due to an increase in the β -cell mass, but the blood glucose level, body weight, and pancreas/body weight ratio were comparable between *Pdx1-Cre;Rb^{f/f}* mice and *Pdx1-Cre* control mice (Fig. 1D; Supplementary Fig. S1E and S1F). The number of Ki67-positive islet cells was higher in *Pdx1-Cre;Rb^{f/f}* mice than in *Pdx1-Cre* control mice at 2 months of age, but equivalent at 4, 6, and 8 months of age (Fig. 1B and E). The ratios of δ -cells and PP cells in the islet cells were comparable between *Pdx1-Cre;Rb^{f/f}* mice and *Pdx1-Cre* control mice (Supplementary Fig. S1G–S1I). Thus, pancreas-specific *Rb* gene deletion does not affect exocrine cells, but exclusively decreases the α -cell/ β -cell ratio in islet cells.

We also examined the effect of *p53* gene mutation on pancreatic tissue by crossing *Pdx1-Cre* mice with *LSL-Trp53^{R172H}* mice (*Pdx1-Cre;Trp53^{R172H}*; Supplementary Fig. S2A). The *Pdx1-Cre;Trp53^{R172H}* mice exhibited no changes in the morphology (Supplementary Fig. S2B), α -cell/ β -cell ratio (Supplementary Fig. S2C), number of Ki67-positive islet cells (Supplementary Fig. S2D), ratio of δ -cells and PP cells in islet cells (Supplementary Fig. S2E and S2F), blood glucose level (Supplementary Fig. S2G), body weight (Supplementary Fig. S2H), or pancreas/body weight ratio (Supplementary Fig. S2I) compared with control mice. In conclusion, *p53* gene mutation does not affect pancreatic development and maintenance.

Pancreas-specific *Rb* gene deletion induces the development of indolent pancreatic neuroendocrine tumors

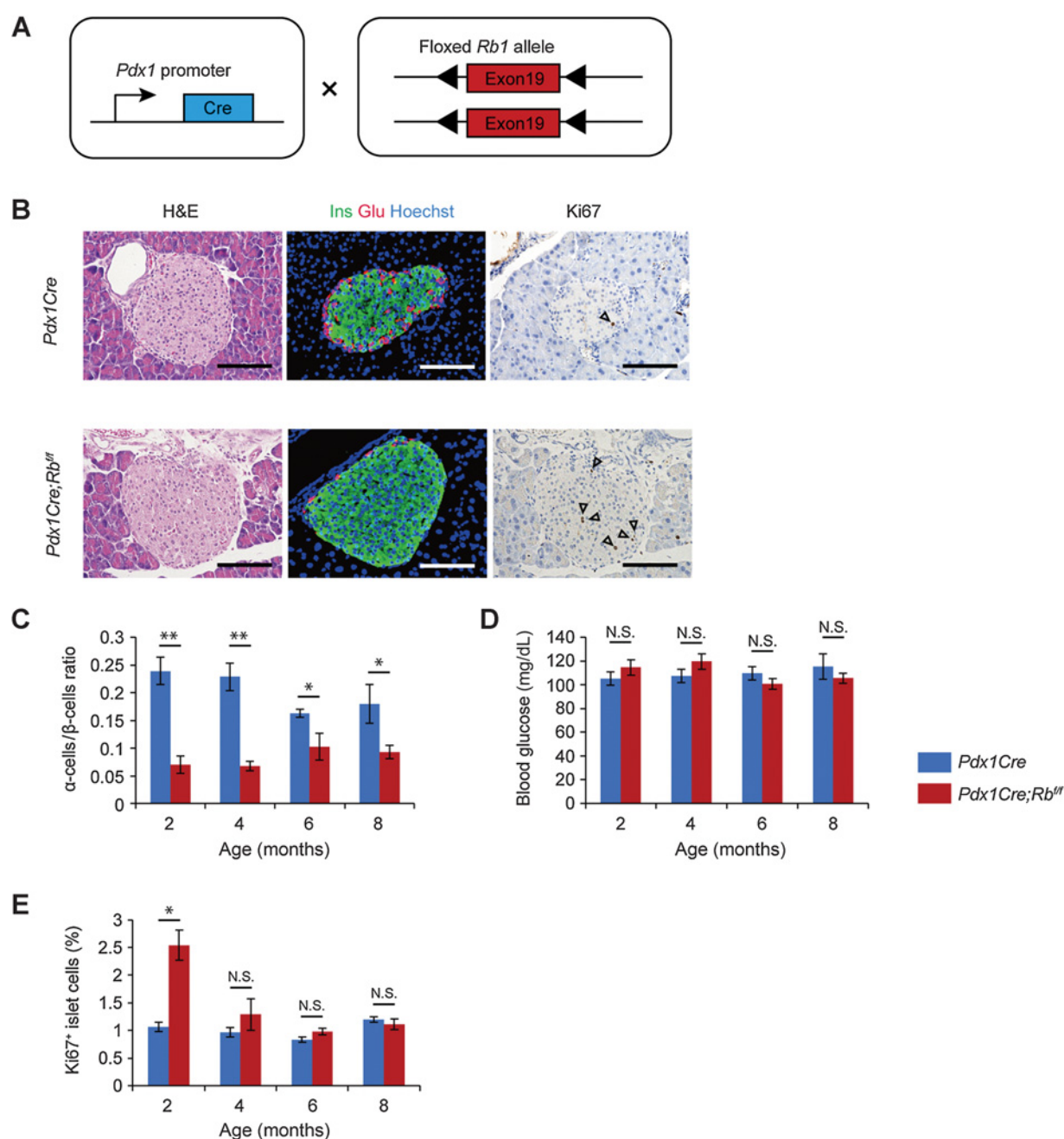
To assess the long-term effect of pancreas-specific *Rb* gene deletion, we compared *Pdx1-Cre;Rb^{f/f}* mice, *Pdx1-Cre* control mice, and *Pdx1-Cre;Trp53^{R172H}* mice at 18 to 20 months of age. No histologic abnormalities were present in the acinar and duct cells in *Pdx1-Cre;Rb^{f/f}* mice, *Pdx1-Cre* control mice, or *Pdx1-Cre;Trp53^{R172H}* mice. In contrast, well-circumscribed PanNETs with a trabecular architecture, dilated abnormal vessels, and apoptotic bodies formed in *Pdx1-Cre;Rb^{f/f}* mice ($N = 4/5$). No tumors were found in *Pdx1-Cre* ($N = 8$) or *Pdx1-Cre;Trp53^{R172H}* ($N = 8$) mice (Fig. 2A). IHC analysis revealed that these tumors were positive for neuroendocrine markers such as chromogranin A and synaptophysin, confirming that the tumors were well-differentiated PanNETs (Fig. 2A). Of seven tumors, 57% (4/7) were positive for PP, 29% (2/7) were positive for glucagon, and 14% (1/7) was positive for somatostatin (Fig. 2A and B). Because of the slow progression and lack of distant metastasis, tumors that formed in the *Pdx1-Cre;Rb^{f/f}* mice were thought to be low grade. These results suggested that pancreas-specific *Rb* deletion, but not *p53* mutation is sufficient to induce low-grade PanNET initiation. Given that the α -cell/ β -cell ratio decreased in islet cells before the development of low-grade PanNETs in *Pdx1-Cre;Rb^{f/f}* mice (Fig. 1B and C), we presumed that the alteration of the α -cell/ β -cell ratio in islet cells was a dysplastic state. The PanNETs in *Pdx1-Cre;Rb^{f/f}* mice were highly penetrant at 18 to 20 months of age (Supplementary Table S1).

p53 is activated in islet cells and PanNETs in pancreas-specific *Rb* deleted mice

To identify major pathways associated with the development of low-grade PanNETs in the presence of *Rb* gene deletion, we performed microarray analysis on pancreatic islets isolated from *Pdx1-Cre* mice and *Pdx1-Cre;Rb^{f/f}* mice. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis showed that the *p53* signaling pathway as well as the cell cycle and DNA replication were among the top five upregulated pathways in pancreatic islets of *Pdx1-Cre;Rb^{f/f}* mice compared with *Pdx1-Cre* control mice (Fig. 3A). We then focused on the *p53* signaling pathway and performed immunohistochemical analysis of pancreatic tissues from *Pdx1-Cre;Rb^{f/f}* mice and *Pdx1-Cre* mice at 18 to 20 months of age. Expression of *p53* was detected in both the islet cells and PanNETs of *Pdx1-Cre;Rb^{f/f}* mice, but not in the islet cells of *Pdx1-Cre* control mice (Fig. 3B). These results indicated that pancreas-specific *Rb* gene deletion induces *p53* activation in islet cells, suggesting the contribution of tumor suppressor *p53* to the indolent phenotype of PanNETs formed in *Pdx1-Cre;Rb^{f/f}* mice.

p53 mutation accelerates the progression of PanNETs in pancreas-specific *Rb* deleted mice

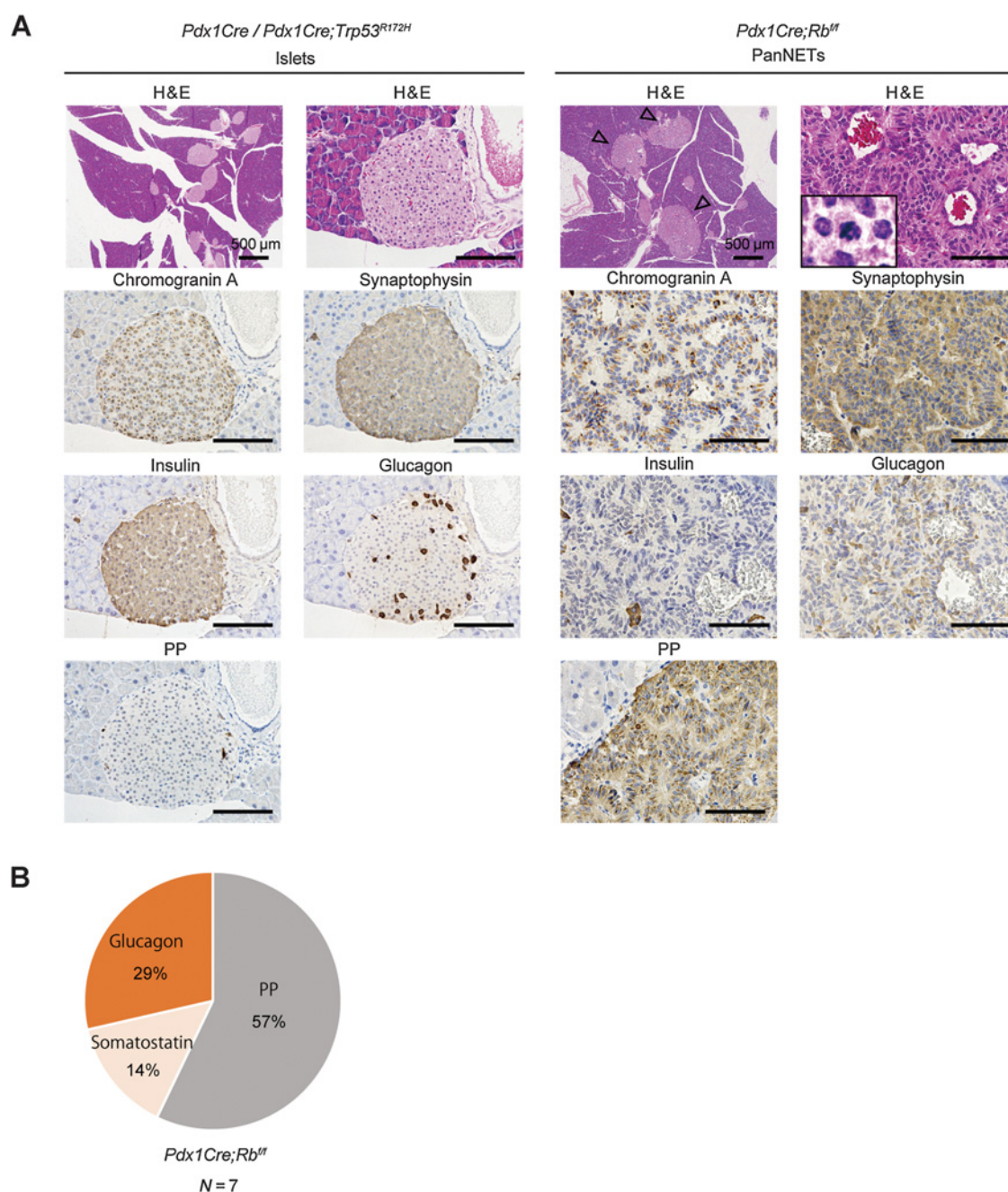
To examine the role of *p53* in pancreas-specific *Rb*-deleted mice, we induced *p53* mutation alleles in the pancreas of *Pdx1-Cre;Rb^{f/f}* mice by crossing them with *LSL-Trp53^{R172H}* mice (*Pdx1-Cre;Trp53^{R172H};Rb^{f/f}*). As observed in *Pdx1-Cre;Rb^{f/f}* mice, the α -cell/ β -cell ratio in islet cells was also significantly lower in *Pdx1-Cre;Trp53^{R172H};Rb^{f/f}* mice than *Pdx1-Cre* control mice at 2 and 4 months of age (Supplementary Fig. S3A and S3B), whereas the exocrine pancreas showed no morphologic changes. In contrast to *Pdx1-Cre;Rb^{f/f}* mice, in *Pdx1-Cre;Trp53^{R172H};Rb^{f/f}* mice, the number of Ki67-positive islet cells was higher than that in *Pdx1-Cre* control mice at both 2 and 4 months of age (Supplementary Fig. S3A and S3C). Moreover, *Pdx1-Cre;Trp53^{R172H};Rb^{f/f}* mice developed slightly enlarged islets with cytological atypia, so-called microadenomas in human, at 2 months (Supplementary Fig. S3D), well-differentiated PanNETs at 4 months, and full penetrance over 6 months (Fig. 4A; Supplementary Table S1).

**Figure 1.**

Influences of Rb deficiency in islet cells. **A**, Experimental strategy for generating the *Pdx1-Cre;Rb^{fl/fl}* mice. Arrow, direction of transcription; arrowheads, *loxP* sites. **B**, Hematoxylin and eosin (H&E), IHC, and immunofluorescence staining of islet cells in *Pdx1-Cre* and *Pdx1-Cre;Rb^{fl/fl}* mice at 2 months of age. Arrowheads, Ki67-positive cells. Scale bars, 100 μ m. **C**, α -Cell/ β -cell ratio in islet cells in *Pdx1-Cre* and *Pdx1-Cre;Rb^{fl/fl}* mice at 2, 4, 6, and 8 months of age ($N = 3-5$). **D**, Blood glucose levels after 4 hours of fasting in *Pdx1-Cre* and *Pdx1-Cre;Rb^{fl/fl}* mice at 2, 4, 6, and 8 months of age ($N = 10-14$). **E**, Ki67⁺ islet cells in *Pdx1-Cre* and *Pdx1-Cre;Rb^{fl/fl}* mice at 2, 4, 6, and 8 months of age ($N = 3-5$). *, $P < 0.05$; **, $P < 0.01$; N.S., nonsignificant.

Notably, some *Pdx1-Cre;Trp53^{R172H};Rb^{fl/fl}* mice had PanNETs with invasive lesions into surrounding tissues (Supplementary Fig. S3E and S3F) and had multiple metastatic lesions in the liver (13.3%, 2/15) at 9 months of age (Fig. 4B). Immunohistochemically, these tumors were positive for neuroendocrine markers such as chromogranin A and synaptophysin, confirming the diagnosis of well-differentiated

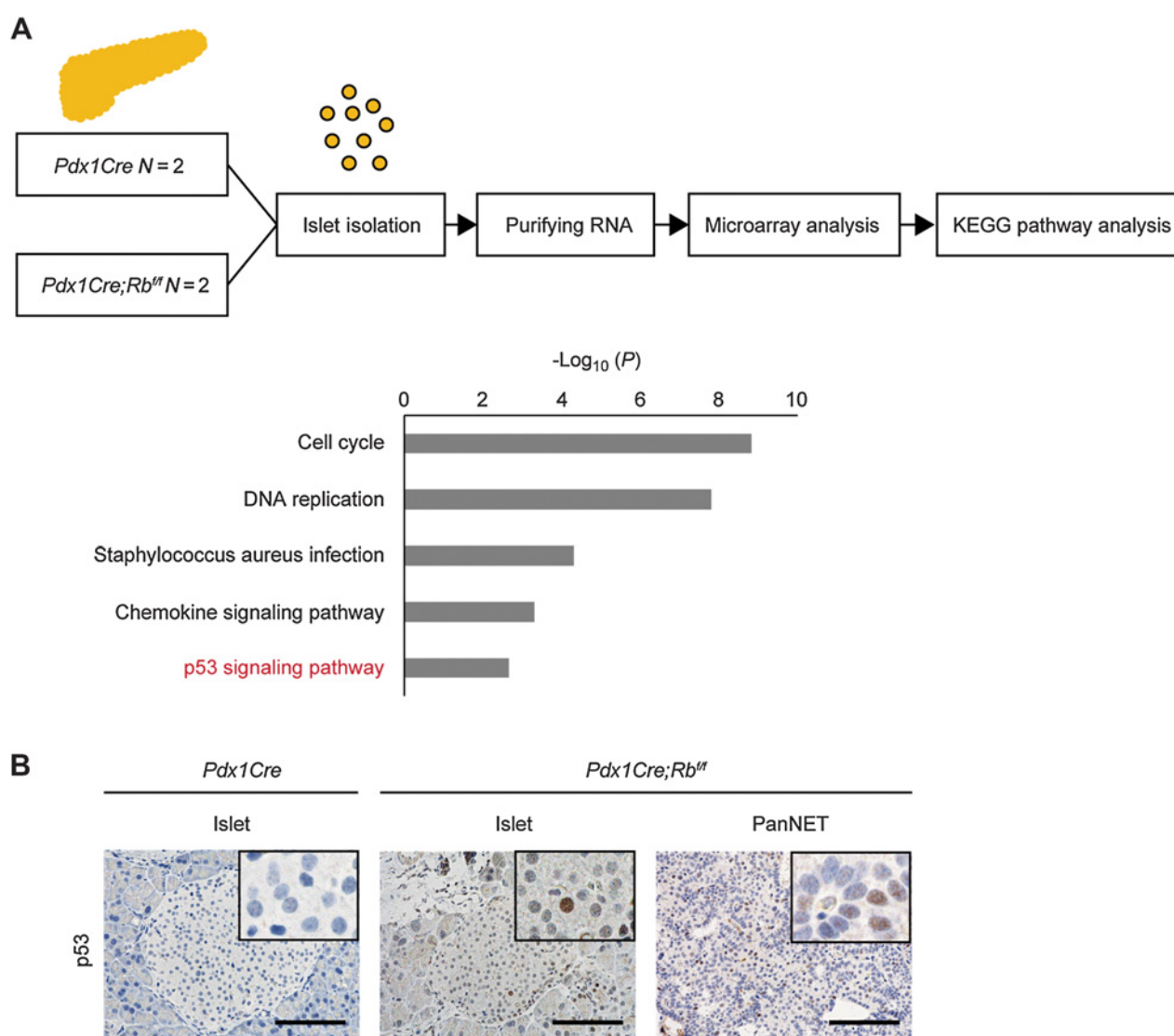
PanNETs as well as tumors in *Pdx1-Cre;Rb^{fl/fl}* mice (Fig. 4A). Regarding hormone production, insulin and glucagon were positive in 76% (26/34) and 24% (8/34; Fig. 4C) of tumors, respectively, but there were no PP-positive or somatostatin-positive tumors. Consistent with the high prevalence of insulinomas, the *Pdx1-Cre;Trp53^{R172H};Rb^{fl/fl}* mice, compared with control mice, had significantly higher plasma insulin

**Figure 2.**

Pancreatic neuroendocrine tumors in *Pdx1-Cre;Rb^{fl}* mice. **A**, Hematoxylin and eosin (H&E) staining and IHC analysis of islet cells in *Pdx1-Cre*, *Pdx1-Cre;Trp53^{R172H}*, and pancreatic tumors in *Pdx1-Cre;Rb^{fl}* mice at 18 to 20 months. Arrowheads, pancreatic neuroendocrine tumors. Scale bars, 100 μ m. **B**, Proportions of different hormone-positive tumors in *Pdx1-Cre;Rb^{fl}* mice observed at 18 to 20 months of age ($N = 7$).

levels and lower glucose levels after the age of 6 months (**Fig. 4D** and **E**). Likely a result of the hypoglycemia, *Pdx1-Cre;Trp53^{R172H};Rb^{fl}* mice had lower body weight (Supplementary Fig. S3G) and a shorter life-span (**Fig. 5A**) than control mice, whereas the pancreas/body weight ratio did not differ between them (Supplementary Fig. S3H). Although the PanNETs that developed in *Pdx1-Cre;Trp53^{R172H};Rb^{fl}* mice were histologically well-differentiated, they were thought to be aggressive tumors due to the rapid tumorigenesis (Supplementary

Table S1), invasiveness to surroundings and formation of liver metastases. Indeed, the PanNETs in *Pdx1-Cre;Trp53^{R172H};Rb^{fl}* mice were relatively larger (**Fig. 5B** and **C**), and the number of Ki67-positive cells was significantly greater (2.7% vs. 24.7%, **Fig. 5D** and **E**) than those in *Pdx1-Cre;Rb^{fl}* mice. In summary, *p53* mutation alone does not affect pancreatic tissue, but in the presence of *Rb* gene deletion, it accelerates tumorigenesis and promotes progression from indolent to aggressive PanNETs.

**Figure 3.**

Upregulation of p53 in islet cells and pancreatic neuroendocrine tumors in *Pdx1-Cre;Rb^{fl/fl}* mice. **A**, Upregulated pathways in *Pdx1-Cre;Rb^{fl/fl}* islet cells compared with *Pdx1-Cre* control islet cells analyzed by microarray analysis with KEGG signaling pathways at 4 to 8 months of age ($N = 2$). **B**, IHC analysis of p53 in *Pdx1-Cre* islet cell and *Pdx1-Cre;Rb^{fl/fl}* islet cell and pancreatic neuroendocrine tumors. Scale bars, 100 μ m.

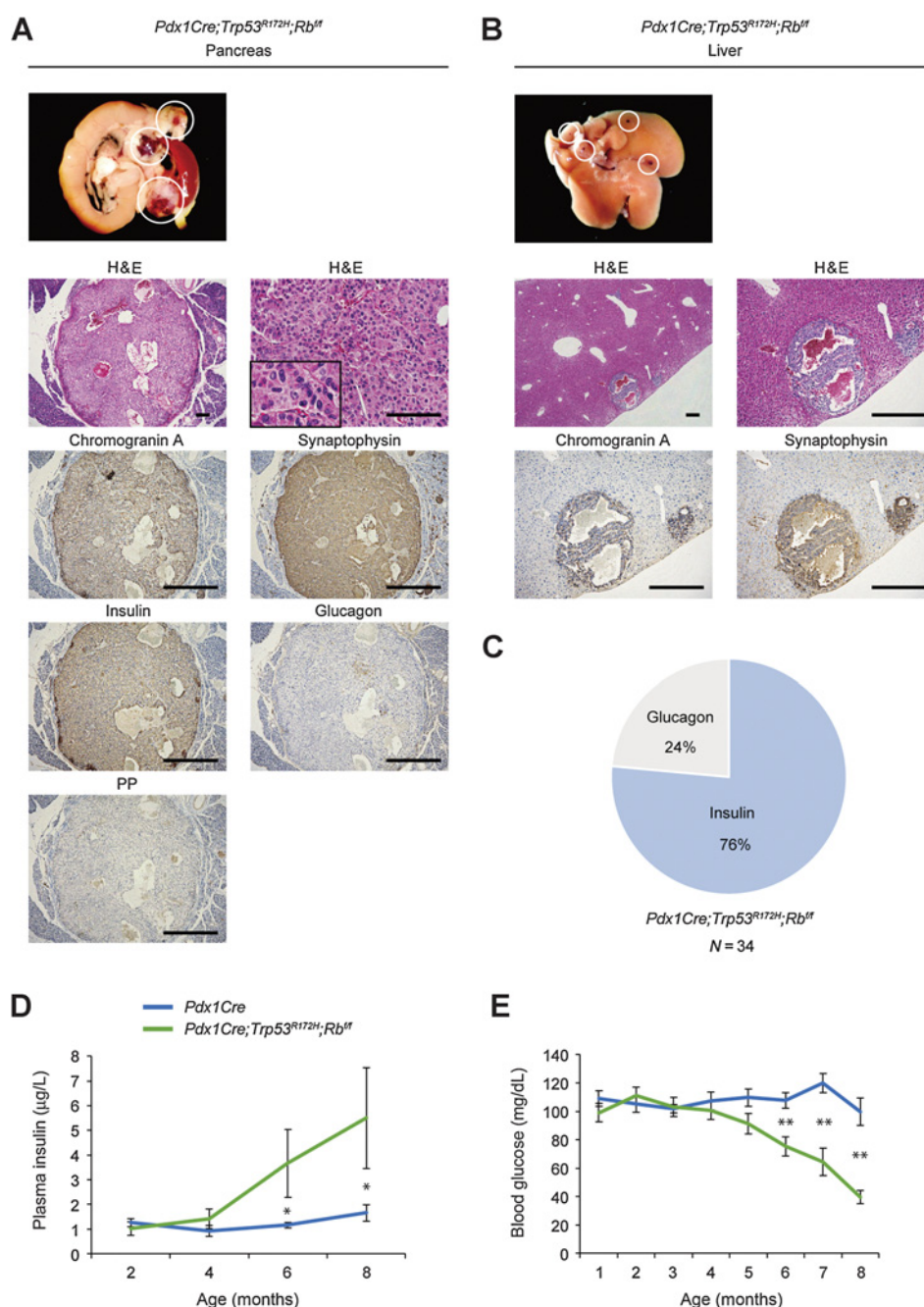
p53 mutation accelerates the progression of PanNETs through mTOR pathway activation

The mTOR pathway is a central pathway in the development of PanNETs in both human and mouse models, including *RIP1-Tag2* mice (24). To assess the activation of the mTOR pathway in our multistep PanNET progression mouse model, we performed IHC analysis of the phosphorylation of S6 ribosomal protein (pS6), which is downstream of mTORC1 (Fig. 6A). We observed a stepwise increase in pS6 from *Pdx1-Cre* control islets to *Pdx1-Cre;Rb^{fl/fl}* PanNETs and *Pdx1-Cre;Trp53^{R172H};Rb^{fl/fl}* PanNETs, suggesting the strong involvement of the mTOR pathway in our mouse model (Fig. 6B). Because p53 suppresses the mTOR pathway through its negative regulators (25) (Fig. 6A), we next examined the mRNA expression of *phosphatase and tensin homolog (Pten)* and *tuberous sclerosis 2 (Tsc2)* by qRT-PCR in normal islet cells isolated from *Pdx1-Cre*, *Pdx1-Cre;Rb^{fl/fl}*, and *Pdx1-*

Cre;Trp53^{R172H};Rb^{fl/fl} mice at the age before forming PanNETs. Expression of *Rb* mRNA was markedly lower in *Pdx1-Cre;Rb^{fl/fl}* and *Pdx1-Cre;Trp53^{R172H};Rb^{fl/fl}* than in *Pdx1-Cre* mice, confirming that these isolated islet cells reflect each genetic background (Fig. 6C). Under these conditions, we found that the expression of *Pten* mRNA and *Tsc2* mRNA in islet cells of *Pdx1-Cre;Trp53^{R172H};Rb^{fl/fl}* was significantly lower than that in *Pdx1-Cre* or *Pdx1-Cre;Rb^{fl/fl}* mice (Fig. 6C). These data suggested that p53 mutation might promote the progression of indolent to aggressive PanNETs, at least in part through mTOR pathway activation via the inhibition of negative regulators.

Discussion

PanNETs are biologically distinct from PanNECs according to the WHO 2017 classification (2–5). Detailed information on precancerous

**Figure 4.**

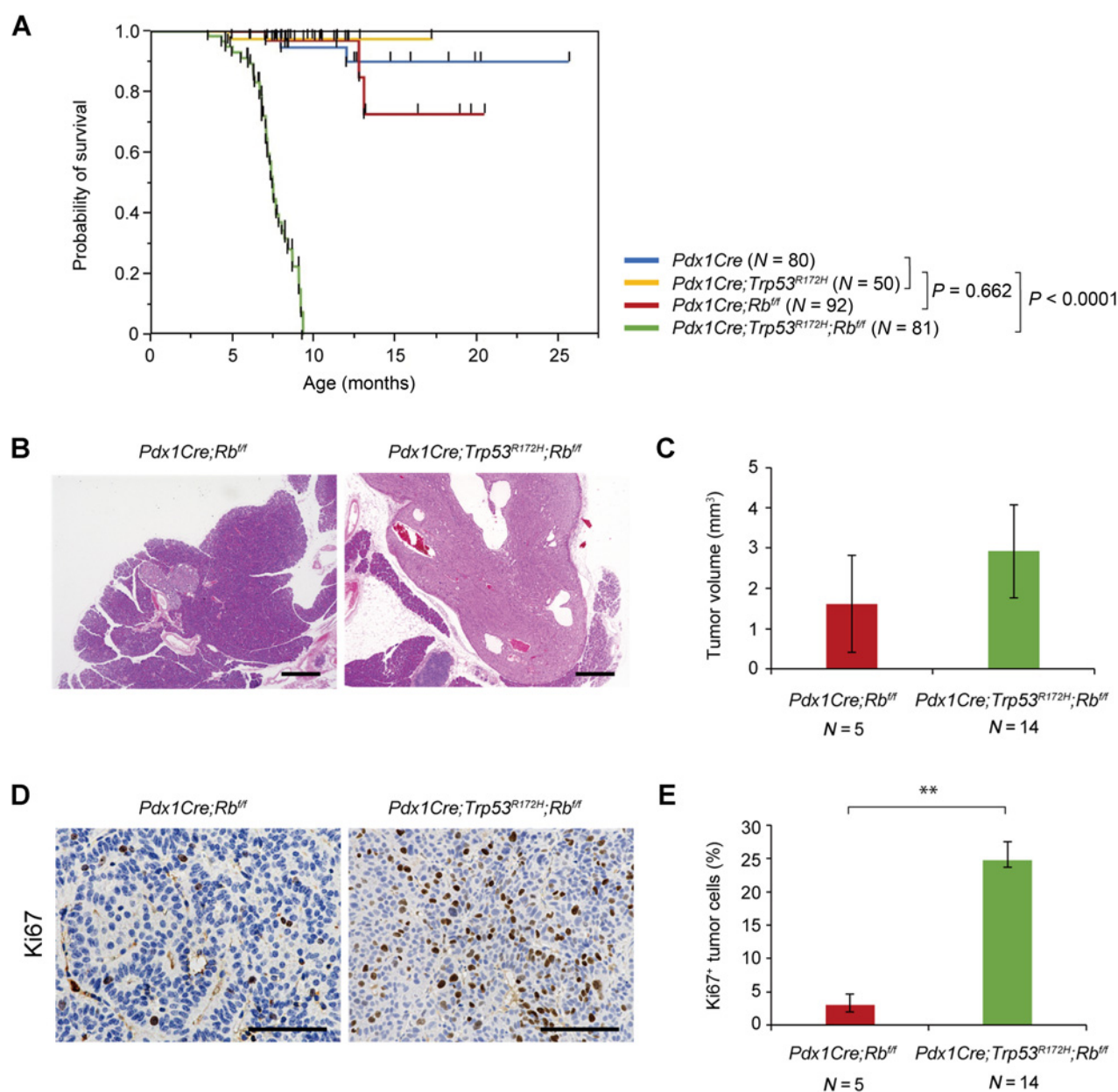
Metastatic pancreatic neuroendocrine tumors in *Pdx1-Cre;Trp53^{R172H};Rb^{fl/f}* mice. **A**, Representative macroscopic image, hematoxylin and eosin (H&E) staining, and IHC staining of pancreatic primary tumors in *Pdx1-Cre;Trp53^{R172H};Rb^{fl/f}* mice at 8 months of age. Scale bars, 100 μm. **B**, Representative macroscopic image, hematoxylin and eosin staining, and IHC staining of liver tumors in *Pdx1-Cre;Trp53^{R172H};Rb^{fl/f}* mice at 9 months of age. Scale bars, 100 μm. **C**, Proportions of different hormone-positive tumors in *Pdx1-Cre;Trp53^{R172H};Rb^{fl/f}* mice observed at 4 to 8 months of age ($N = 34$). **D**, Plasma insulin levels in *Pdx1-Cre* control mice and *Pdx1-Cre;Trp53^{R172H};Rb^{fl/f}* mice at 2, 4, 6, and 8 months of age ($N = 4-8$). **E**, Blood glucose levels after 4 hours fasting in *Pdx1-Cre* control mice and *Pdx1-Cre;Trp53^{R172H};Rb^{fl/f}* mice at 1 to 8 months of age ($N = 7-14$).

lesions of PanNETs or the mechanisms of their progression, however, has been lacking. In this study, by manipulating the *Rb* and *p53* genes, we established a multistep progression model from dysplastic islets to indolent PanNETs and aggressive metastatic PanNETs in mice.

A previous report demonstrated that the β -cell mass is increased and the α -cell/ β -cell ratio is decreased in islet cells in *Pdx1-Cre;Rb^{fl/f}* mice, leading to the acquisition of resistance to diabetes (26). In that report, the authors reported that pancreas-specific *Rb* ablation affected α -cell differentiation and converted the α -cell/ β -cell ratio during their observation period of 5.5 months (26). We observed almost the same phenotype in our *Pdx1-Cre;Rb^{fl/f}* mice (Fig. 1); however, in our long-term observation of 20 months, we observed the development of

histologically confirmed indolent PanNETs with a Ki67 index of 2.7% in *Pdx1-Cre;Rb^{fl/f}* mice, at high penetrance (Fig. 2). To the best of our knowledge, this is the first report demonstrating the formation of PanNETs by *Rb* gene deletion alone. On the basis of our sequential and long-term observations, the early phenotype of a decreased α -cell/ β -cell ratio after *Rb* gene deletion might be part of the dysplastic state before the formation of microadenomas or PanNETs. Interestingly, although the genetic manipulation was present in all pancreatic epithelial cells, the *Rb* gene deletion exclusively affected islet cells and not exocrine cells. This observation contrasts with the previous finding that pancreas-specific induction of a *Kras* gene mutation affects only exocrine cells and not islet cells (17). Pancreatic epithelial cells may be

Roles of Rb and p53 in Pancreatic Neuroendocrine Tumors

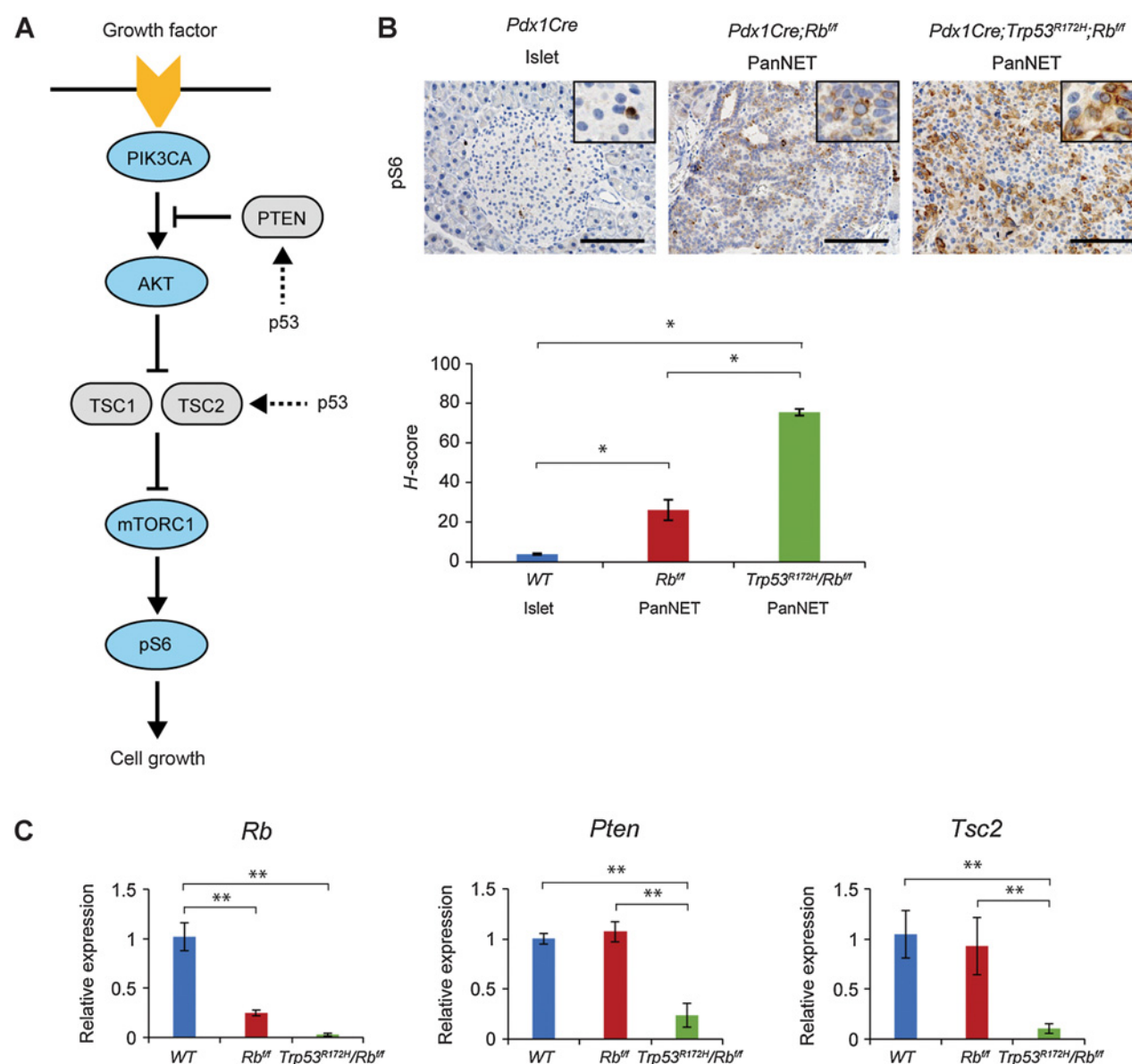
**Figure 5.**

Comparisons of tumors in *Pdx1-Cre;Rb^{fl/fl}* and *Pdx1-Cre;Trp53^{R172H};Rb^{fl/fl}* mice. **A**, Kaplan-Meier survival curve for each genotype. **B**, Hematoxylin and eosin staining in PanNETs in *Pdx1-Cre;Rb^{fl/fl}* mice at 18 months of age and *Pdx1-Cre;Trp53^{R172H};Rb^{fl/fl}* mice at 8 months of age. Scale bars, 500 μ m. **C**, Tumor volumes of *Pdx1-Cre;Rb^{fl/fl}* (N = 5) and *Pdx1-Cre;Trp53^{R172H};Rb^{fl/fl}* mice (N = 14). **D**, Immunohistochemical staining of Ki67 in PanNETs of *Pdx1-Cre;Rb^{fl/fl}* and *Pdx1-Cre;Trp53^{R172H};Rb^{fl/fl}* mice. Scale bars, 100 μ m. **E**, Ki67-positive tumor cells in *Pdx1-Cre;Rb^{fl/fl}* (N = 5) and *Pdx1-Cre;Trp53^{R172H};Rb^{fl/fl}* (N = 14) mice. **, $P < 0.01$.

susceptible to oncogenes in a cell context-dependent manner. Thus, we concluded that pancreas-specific *Rb* gene deletion is sufficient for the development of PanNETs in mice. This concept was further supported by reports that *Rb* is in the same molecular pathway of cell-cycle regulation and tumor suppression as *Men1* (27, 28), which is involved in PanNETs formation in both humans and mice.

In contrast to the *Rb* gene deletion, the induction of a pancreas-specific *p53* mutation alone did not affect pancreatic cells, even after long-term observation. This is compatible with the previous reports of *PHLDA3*, a target gene of *p53* and a suppressor of Akt-mTOR

pathway; *PHLDA3*-deficient mice develop hyperplastic islets but do not develop PanNETs (29). However, *wild-type p53* activation was observed at the mRNA and protein levels in islet cells of *Pdx1-Cre;Rb^{fl/fl}* mice, suggesting that activated *p53* suppresses tumor progression in response to *Rb* gene deletion (Fig. 3). This hypothesis was confirmed by the observation that *Pdx1-Cre;Trp53^{R172H};Rb^{fl/fl}* mice developed well-differentiated, but aggressive PanNETs with a high Ki67 index of 24.7% within only 4 months (Figs. 4 and 5). Furthermore, these mice had liver metastases in 13.3%, although the incidence was lower than in previous reports of *RIP1-Tag2* mice (30). In these mice, we

**Figure 6.**

Stepwise upregulation of mTOR pathway in *Pdx1-Cre;Rb^{f/f}* and *Pdx1-Cre;Trp53^{R172H};Rb^{f/f}* islet cells and PanNETs. **A**, Schematic diagram including p53 target genes that repress the mTOR signaling pathway identified in this study. **B**, Activation of the mTOR pathway, analyzed according to H-score of pS6 expression in *Pdx1-Cre* islets, *Pdx1-Cre;Rb^{f/f}* PanNETs, and *Pdx1-Cre;Trp53^{R172H};Rb^{f/f}* PanNETs by immunohistochemistry. Scale bars, 100 μ m. The H-score of pS6 expression increased in a stepwise manner among these cells. **C**, qRT-PCR in isolated islet cells in *Pdx1-Cre* ($N = 3$), *Pdx1-Cre;Rb^{f/f}* ($N = 3$), and *Pdx1-Cre;Trp53^{R172H};Rb^{f/f}* ($N = 4$) mice at 3 to 8 months of age. *, $P < 0.05$; **, $P < 0.01$.

reasoned that PanNETs initiated by *Rb* gene deletion were released from tumor suppression through the induction of a *p53* mutation and then progressed to aggressive tumors. This observation corresponds to the finding that PanIN initiated by a *Kras* mutation progresses to invasive pancreatic ductal adenocarcinoma through the induction of a *p53* mutation in *Pdx1-Cre;Trp53^{R172H};Kras^{G12D}* (so-called KPC) mice. Thus, *p53* itself does not affect tumor initiation in pancreatic tissue but appears to be involved in the progression of endocrine tumors and exocrine tumors initiated by the *Rb* and *Kras* genes, respectively.

In human PanNETs, genes in the mTOR pathway are frequently mutated, and thus inhibitors of mTOR significantly improve survival.

Loss of heterozygosity for the aforementioned PHLDA3 has been reported in 72% of human PanNET (29). Moreover, the expression of mTOR pathway components in various human NETs is predictive of the prognosis. For example, the activation of downstream targets of mTOR pathway such as p-RPS6KB1 or p-RPS6 is associated with a poor prognosis (31). Decreased expression of TSC2 or PTEN, which are negative regulators of the mTOR pathway, is significantly associated with shorter disease-free survival and overall survival (32). Notably, in our new mouse model, the mRNA expression of *Pten* and *Tsc2* in the islet cells of *Pdx1-Cre;Trp53^{R172H};Rb^{f/f}* mice was significantly decreased, and the expression of pS6, a downstream component of the

mTOR pathway, was upregulated in a stepwise manner in *Pdx1-Cre; Rb^{fl/fl}* mice and *Pdx1-Cre; Trp53^{R172H}; Rb^{fl/fl}* mice compared with control mice. From these observations, we speculate that activation of the mTOR pathway through the regulation of TSC2 and PTEN by p53 plays a major role in switching the progression of indolent to aggressive PanNETs.

Genetic alterations in *Rb* and *TP53* have been demonstrated to be uncommon in large-scale genome analysis of human PanNETs (6, 7), rather *Rb* loss and *KRAS* mutation have been shown in 54.5% and 48.7% of human PanNECs, respectively (5). Therefore, it is worth discussing why *Rb* gene deletion does not reflect human disease and forms PanNET in our mouse model. There may be several reasons for this. Given the high prevalence of *KRAS* mutations in human PanNECs, *Kras* mutations may be required in addition to *Rb* deletion to form PanNECs in mice. Because of cells of origin can be intrinsically different between PanNEC and PanNET (33, 34), mouse PanNEC may only be reproduced when the *Kras* and *Rb* abnormalities are introduced in the proper cells in the proper order. On the other hand, there are possible causes for the formation of PanNETs in mice by *Rb* abnormalities. For example, there have been reports of copy-number alteration (35), mutation (8), and upstream abnormalities of *Rb* pathway including *CDK4/6* (10, 11), *p27* (36, 37), *RABL6A* (38, 39) in human PanNETs. Thus, abnormalities of *Rb* pathway other than *Rb* gene alteration may be involved in PanNETs. Recently, the *CDK4/6* inhibitors, palbociclib, commonly used in breast cancer, are trialed in PanNETs (40). Combination therapies including *CDK4/6* inhibitors that is based on preclinical studies in other cancers (41) may be an effective treatment option in the future. In this point of view, further development of clinical trials evaluating *Rb* pathway including *CDK4/6* inhibitors is eagerly awaited. As for *TP53*, its genetic alterations are not common in human PanNETs but still observed in a few cases (6, 7, 42). Quite interestingly, it has been suggested that *TP53* mutations are more likely found in G3 PanNETs than in G1/2 PanNETs (7, 42) and may be associated with metastasis (43). Together with the results of our mouse model, these previous reports may suggest that *p53* mutations are involved in tumor progression of PanNETs as a late event. Thus, the mouse model we have newly established has several genetic aspects that do not correspond to those of humans. However, this model recapitulates the stepwise progression to G3 PanNET, which could not have been sufficiently studied so far, and may provide new insights into the pathogenesis of PanNETs. This model is expected to open the way to new diagnostic and therapeutic

approach for PanNETs and to be a useful tool for future preclinical studies.

In summary, our novel mouse model demonstrated that inactivation of *Rb* in the pancreas affects islet cells exclusively and is sufficient for the development of dysplastic islet cells and the subsequent formation of indolent PanNETs. In contrast, *p53* mutation itself has no effect on PanNET initiation, but plays a crucial role in the progression from indolent to aggressive PanNETs through activation of the mTOR pathway in mice. On the basis of these findings, we conclude that *Rb* and *p53* have distinct roles in the initiation and progression, respectively, of PanNETs.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Y. Yamauchi: Conceptualization, resources, data curation, software, formal analysis, validation, investigation, visualization, methodology and writing-original draft. **Y. Kodama:** Conceptualization, funding acquisition, writing-original draft, project administration, writing-review and editing. **M. Shiokawa:** Data curation. **N. Kakiuchi:** Data curation. **S. Marui:** Conceptualization. **T. Kuwada:** Conceptualization. **Y. Sogabe:** Conceptualization. **T. Tomono:** Conceptualization. **A. Mima:** Conceptualization. **T. Morita:** Conceptualization. **T. Matsumori:** Conceptualization. **T. Ueda:** Conceptualization. **M. Tsuda:** Conceptualization. **Y. Nishikawa:** Conceptualization. **K. Kuriyama:** Conceptualization. **Y. Sakuma:** Conceptualization. **Y. Ota:** Conceptualization. **T. Maruno:** Conceptualization. **N. Uza:** Conceptualization. **A. Masuda:** Resources. **H. Tatsuoka:** Investigation. **D. Yabe:** Conceptualization. **S. Minamiguchi:** Investigation. **T. Masui:** Resources. **N. Inagaki:** Conceptualization. **S. Uemoto:** Resources. **T. Chiba:** Supervision and funding acquisition. **H. Seno:** Supervision and funding acquisition.

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References

1. Yao JC, Hassan M, Phan A, Dagohoy C, Leary C, Mares JE, et al. One hundred years after "carcinoid": epidemiology of and prognostic factors for neuroendocrine tumors in 35,825 cases in the United States. *J Clin Oncol* 2008;26:3063–72.
2. Yachida S, Vakiani E, White CM, Zhong Y, Saunders T, Morgan R, et al. Small cell and large cell neuroendocrine carcinomas of the pancreas are genetically similar and distinct from well-differentiated pancreatic neuroendocrine tumors. *Am J Surg Pathol* 2012;36:173–84.
3. Basturk O, Tang L, Hruban RH, Adsay V, Yang Z, Krasinskas AM, et al. Poorly differentiated neuroendocrine carcinomas of the pancreas: a clinicopathologic analysis of 44 cases. *Am J Surg Pathol* 2014;38:437–47.
4. Tang LH, Basturk O, Sue JJ, Klimstra DS. A practical approach to the classification of WHO grade 3 (G3) well-differentiated neuroendocrine tumor (WDNET) and poorly differentiated neuroendocrine carcinoma (PD-NEC) of the pancreas. *Am J Surg Pathol* 2016;40:1192–202.
5. Hijioka S, Hosoda W, Matsuo K, Ueno M, Furukawa M, Yoshitomi H, et al. *Rb* Loss and *KRAS* mutation are predictors of the response to platinum-based chemotherapy in pancreatic neuroendocrine neoplasm with grade 3: a Japanese Multicenter Pancreatic NEN-G3 Study. *Clin Cancer Res* 2017;23:4625–32.
6. Jiao Y, Shi C, Edil BH, de Wilde RF, Klimstra DS, Maitra A, et al. DAXX/ATRX, MEN1, and mTOR pathway genes are frequently altered in pancreatic neuroendocrine tumors. *Science* 2011;331:1199–203.
7. Scarpa A, Chang DK, Nones K, Corbo V, Patch AM, Bailey P, et al. Whole-genome landscape of pancreatic neuroendocrine tumours. *Nature* 2017;543:65–71.
8. Vijayvergia N, Boland PM, Handorf E, Gustafson KS, Gong Y, Cooper HS, et al. Molecular profiling of neuroendocrine malignancies to identify prognostic and therapeutic markers: a Fox Chase Cancer Center Pilot Study. *Br J Cancer* 2016;115:564–70.
9. Liu H, Dibling B, Spike B, Dirlam A, Macleod K. New roles for the RB tumor suppressor protein. *Curr Opin Genet Dev* 2004;14:55–64.
10. Tang LH, Contractor T, Clausen R, Klimstra DS, Du YC, Allen PJ, et al. Attenuation of the retinoblastoma pathway in pancreatic neuroendocrine tumors due to increased cdk4/cdk6. *Clin Cancer Res* 2012;18:4612–20.
11. Shi Y, Qian ZR, Zhang S, Li W, Masugi Y, Li T, et al. Cell cycle protein expression in neuroendocrine tumors: association of CDK4/CDK6, CCND1, and phosphorylated retinoblastoma protein with proliferative index. *Pancreas* 2017;46:1347–53.

12. Hu W, Feng Z, Modica I, Klimstra DS, Song L, Allen PJ, et al. Gene amplifications in well-differentiated pancreatic neuroendocrine tumors inactivate the p53 pathway. *Genes Cancer* 2010;1:360–8.
13. Hanahan D. Heritable formation of pancreatic beta-cell tumours in transgenic mice expressing recombinant insulin/simian virus 40 oncogenes. *Nature* 1985; 315:115–22.
14. Efrat S, Teitelman G, Anwar M, Ruggiero D, Hanahan D. Glucagon gene regulatory region directs oncoprotein expression to neurons and pancreatic alpha cells. *Neuron* 1988;1:605–13.
15. Lee YC, Asa SL, Drucker DJ. Glucagon gene 5'-flanking sequences direct expression of simian virus 40 large T antigen to the intestine, producing carcinoma of the large bowel in transgenic mice. *J Biol Chem* 1992;267: 10705–8.
16. Glenn ST, Jones CA, Sexton S, LeVea CM, Caraker SM, Hajduczk G, et al. Conditional deletion of p53 and Rb in the renin-expressing compartment of the pancreas leads to a highly penetrant metastatic pancreatic neuroendocrine carcinoma. *Oncogene* 2014;33:5706–15.
17. Hingorani SR, Petricoin EF, Maitra A, Rajapakse V, King C, Jacobetz MA, et al. Preinvasive and invasive ductal pancreatic cancer and its early detection in the mouse. *Cancer Cell* 2003;4:437–50.
18. Srinivas S, Watanabe T, Lin CS, William CM, Tanabe Y, Jessell TM, et al. Cre reporter strains produced by targeted insertion of EYFP and ECFP into the ROSA26 locus. *BMC Dev Biol* 2001;1:4.
19. Marino S, Vooijs M, van Der Gulden H, Jonkers J, Berns A. Induction of medulloblastomas in p53-null mutant mice by somatic inactivation of Rb in the external granular layer cells of the cerebellum. *Genes Dev* 2000;14:994–1004.
20. Olive KP, Tuveson DA, Ruhe ZC, Yin B, Willis NA, Bronson RT, et al. Mutant p53 gain of function in two mouse models of Li-Fraumeni syndrome. *Cell* 2004; 119:847–60.
21. Olivier M, Hussain SP, Caron de Fromentel C, Hainaut P, Harris CC. TP53 mutation spectra and load: a tool for generating hypotheses on the etiology of cancer. *IARC Sci Publ* 2004;247–70.
22. Sion-Vardy N, Freedman J, Lazarov I, Bolotin A, Ariad S. p27kip1 Expression in non-small cell lung cancer is not an independent prognostic factor. *Anticancer Res* 2010;30:3699–704.
23. Sutton R, Peters M, McShane P, Gray DW, Morris PJ. Isolation of rat pancreatic islets by ductal injection of collagenase. *Transplantation* 1986;42:689–91.
24. Chiu CW, Nozawa H, Hanahan D. Survival benefit with proapoptotic molecular and pathologic responses from dual targeting of mammalian target of rapamycin and epidermal growth factor receptor in a preclinical model of pancreatic neuroendocrine carcinogenesis. *J Clin Oncol* 2010;28:4425–33.
25. Akeno N, Miller AL, Ma X, Wikenheiser-Brokamp KA. p53 suppresses carcinoma progression by inhibiting mTOR pathway activation. *Oncogene* 2015;34: 589–99.
26. Cai EP, Wu X, Schroer SA, Elia AJ, Nostro MC, Zacksenhaus E, et al. Retinoblastoma tumor suppressor protein in pancreatic progenitors controls alpha- and beta-cell fate. *Proc Natl Acad Sci U S A* 2013;110:14723–8.
27. Matoso A, Zhou Z, Hayama R, Flesken-Nikitin A, Nikitin AY. Cell lineage-specific interactions between Men1 and Rb in neuroendocrine neoplasia. *Carcinogenesis* 2008;29:620–8.
28. Loffler KA, Biondi CA, Gartside MG, Serewko-Auret MM, Duncan R, Tonks ID, et al. Lack of augmentation of tumor spectrum or severity in dual heterozygous Men1 and Rb1 knockout mice. *Oncogene* 2007;26:4009–17.
29. Ohki R, Saito K, Chen Y, Kawase T, Hiraoka N, Saigawa R, et al. PHLDA3 is a novel tumor suppressor of pancreatic neuroendocrine tumors. *Proc Natl Acad Sci U S A* 2014;111:E2404–13.
30. Paez-Ribes M, Allen E, Hudock J, Takeda T, Okuyama H, Vinals F, et al. Antiangiogenic therapy elicits malignant progression of tumors to increased local invasion and distant metastasis. *Cancer Cell* 2009;15:220–31.
31. Qian ZR, Ter-Minassian M, Chan JA, Imamura Y, Hooshmand SM, Kuchiba A, et al. Prognostic significance of mTOR pathway component expression in neuroendocrine tumors. *J Clin Oncol* 2013;31:3418–25.
32. Missiaglia E, Dalai I, Barbi S, Beghelli S, Falconi M, della Peruta M, et al. Pancreatic endocrine tumors: expression profiling evidences a role for AKT-mTOR pathway. *J Clin Oncol* 2010;28:245–55.
33. Schimmack S, Svejda B, Lawrence B, Kidd M, Modlin IM. The diversity and commonalities of gastroenteropancreatic neuroendocrine tumors. *Langenbecks Arch Surg* 2011;396:273–98.
34. Kloppel G. Neuroendocrine neoplasms: dichotomy, origin and classifications. *Visc Med* 2017;33:324–30.
35. Williamson LM, Steel M, Grewal JK, Thibodeau ML, Zhao EY, Loree JM, et al. Genomic characterization of a well-differentiated grade 3 pancreatic neuroendocrine tumor. *Cold Spring Harb Mol Case Stud* 2019;5:a003814.
36. Grabowski P, Schrader J, Wagner J, Horsch D, Arnold R, Arnold CN, et al. Loss of nuclear p27 expression and its prognostic role in relation to cyclin E and p53 mutation in gastroenteropancreatic neuroendocrine tumors. *Clin Cancer Res* 2008;14:7378–84.
37. Kim HS, Lee HS, Nam KH, Choi J, Kim WH. p27 loss is associated with poor prognosis in gastroenteropancreatic neuroendocrine tumors. *Cancer Res Treat* 2014;46:383–92.
38. Umesalma S, Kaemmer CA, Kohlmeyer JL, Letney B, Schab AM, Reilly JA, et al. RABL6A inhibits tumor-suppressive PP2A/AKT signaling to drive pancreatic neuroendocrine tumor growth. *J Clin Invest* 2019;130:1641–53.
39. Hagen J, Muniz VP, Falls KC, Reed SM, Taghiyev AF, Quelle FW, et al. RABL6A promotes G1-S phase progression and pancreatic neuroendocrine tumor cell proliferation in an Rb1-dependent manner. *Cancer Res* 2014;74:6661–70.
40. Grande E, Teule A, Alonso-Gordoa T, Jimenez-Fonseca P, Benavent M, Capdevila J, et al. The PALBONET trial: a phase II study of palbociclib in metastatic grade 1 and 2 pancreatic neuroendocrine tumors (GETNE-1407). *Oncologist* 2020 [Epub ahead of print].
41. Ruscetti M, Leibold J, Bott MJ, Fennell M, Kulick A, Salgado NR, et al. NK cell-mediated cytotoxicity contributes to tumor control by a cytostatic drug combination. *Science* 2018;362:1416–22.
42. Konukiewitz B, Jesinghaus M, Steiger K, Schlitter AM, Kasajima A, Sipos B, et al. Pancreatic neuroendocrine carcinomas reveal a closer relationship to ductal adenocarcinomas than to neuroendocrine tumors G3. *Hum Pathol* 2018;77:70–9.
43. Weisbrod AB, Zhang L, Jain M, Barak S, Quezado MM, Kebebew E. Altered PTEN, ATRX, CHGA, CHGB, and TP53 expression are associated with aggressive VHL-associated pancreatic neuroendocrine tumors. *Horm Cancer* 2013;4: 165–75.

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Rb and p53 Execute Distinct Roles in the Development of Pancreatic Neuroendocrine Tumors

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Supplemental Table 1. PanNETs incidence per genotype

Age	<i>Pdx1Cre</i>	<i>Pdx1Cre;Trp53^{R172H}</i>	<i>Pdx1Cre;Rb^{ff}</i>	<i>Pdx1Cre;Trp53^{R172H};Rb^{ff}</i>
2 months	0/5 (0%)	0/4 (0%)	0/4 (0%)	0/5 (0%)
4 months	0/5 (0%)	0/3 (0%)	0/5 (0%)	1/5 (20%)
6 months	0/4 (0%)	0/4 (0%)	0/4 (0%)	8/8 (100%)
8 months	0/4 (0%)	0/4 (0%)	0/4 (0%)	6/6 (100%)
18 - 20 months	0/8 (0%)	0/8 (0%)	4/5 (80%)	-

Figure. S1

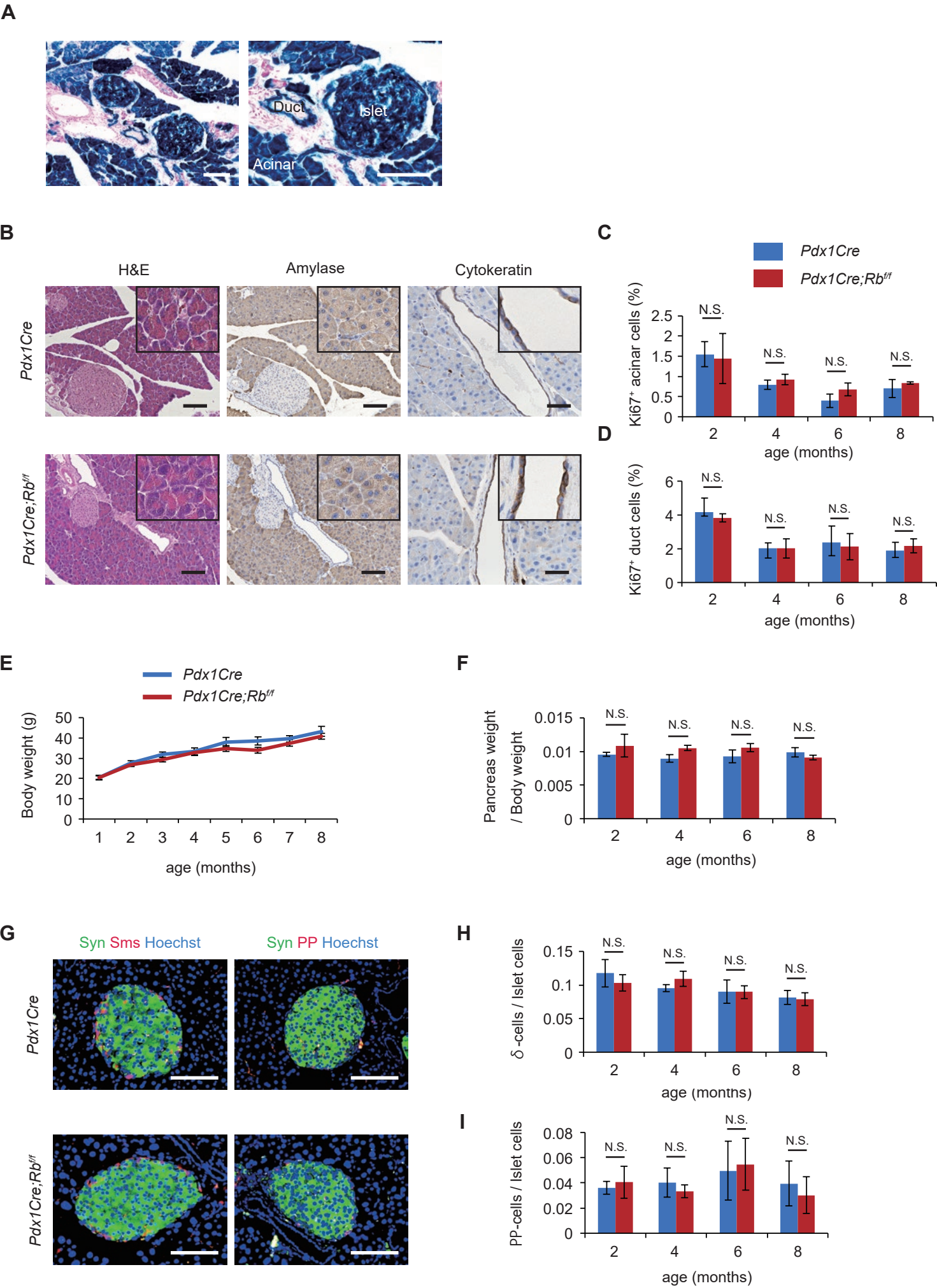
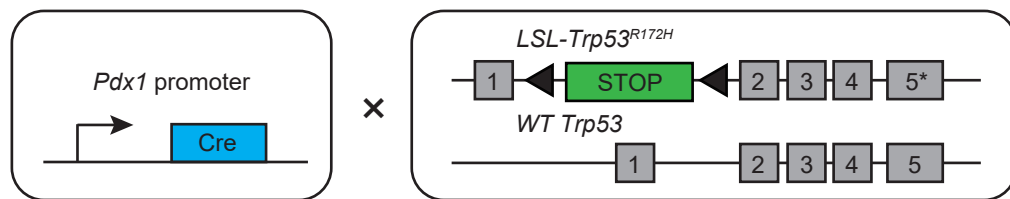
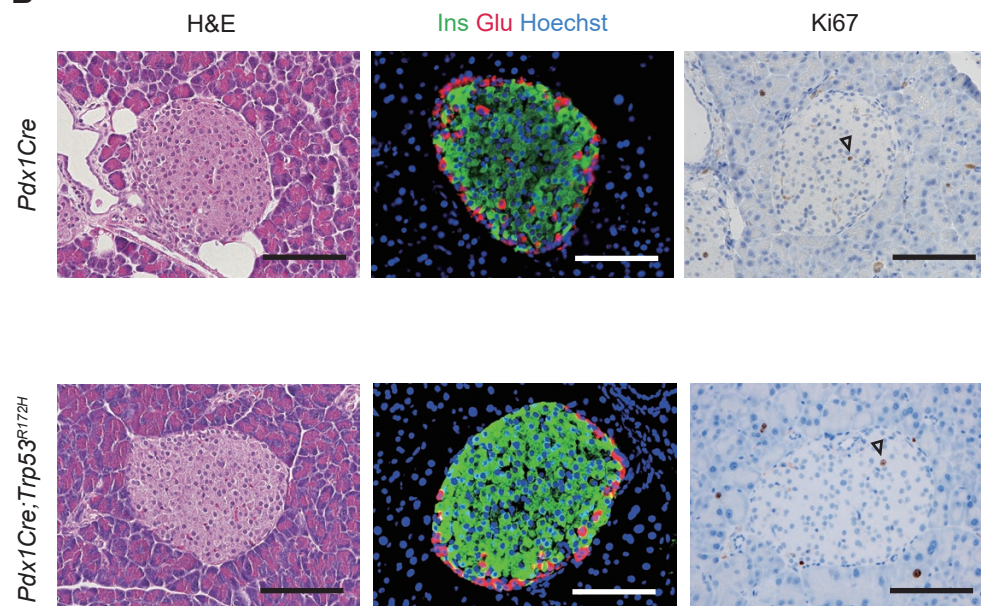


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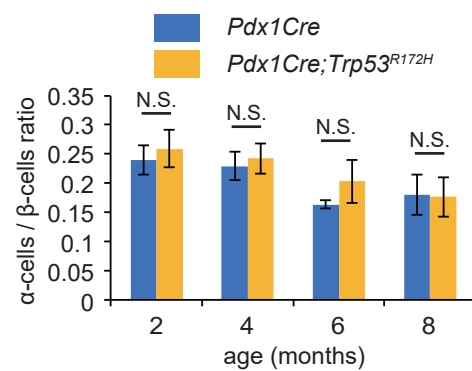
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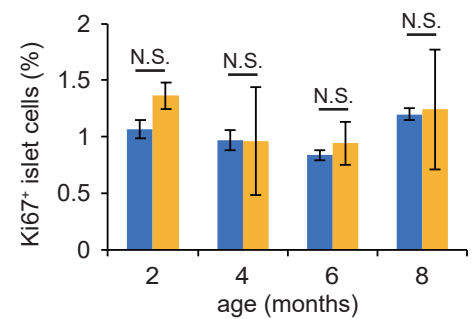
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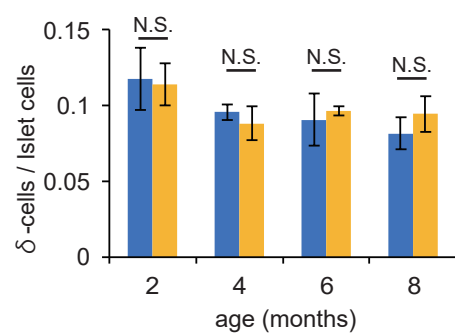
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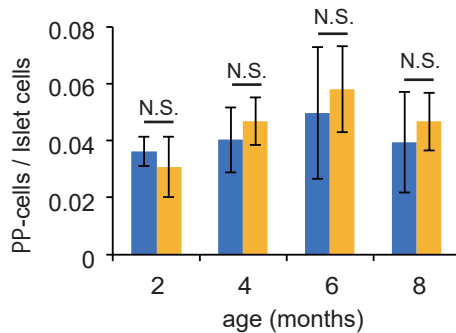
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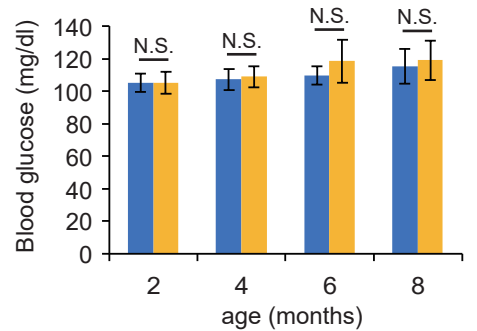
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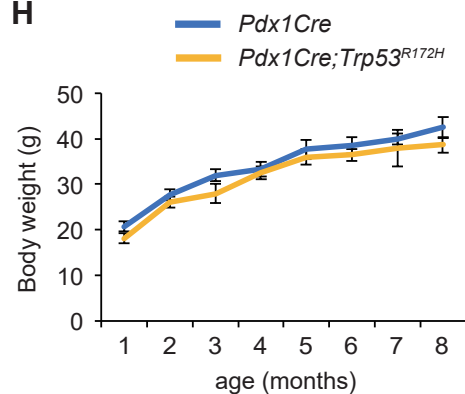
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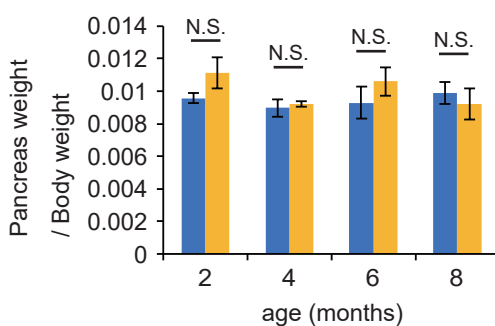
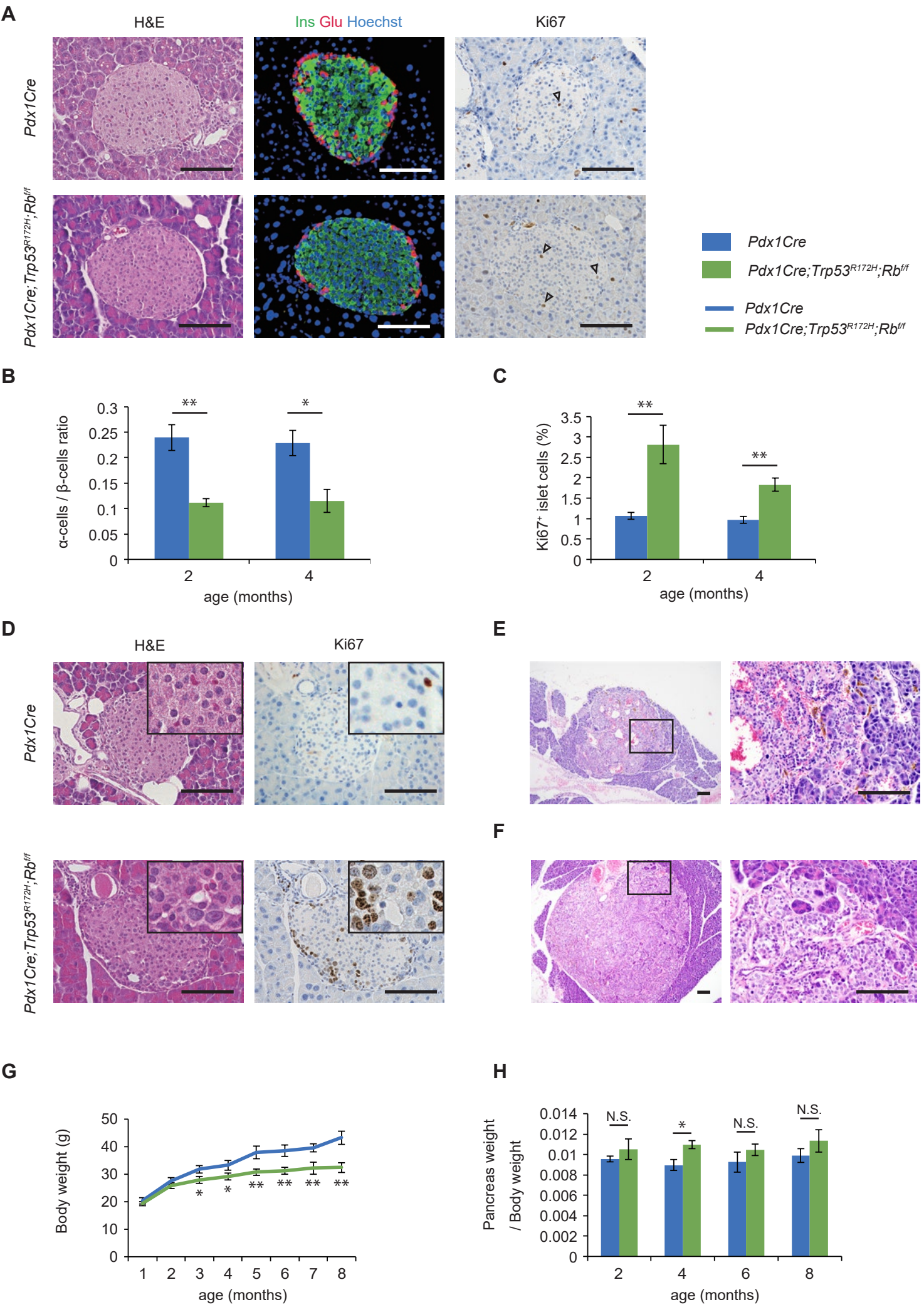


Figure. S3



1 **Supplemental information**

2 **Supplemental Figure legends**

3

4 **Supplemental Table 1.**

5 Incidence of PanNETs formation.

6

7 **Supplemental Figure 1.**

8 A, Representative images of LacZ staining in the pancreas in *Pdx1-Cre;Rosa26R* mice.

9 Scale bars: 100 μ m. B, H&E staining and immunohistochemical staining for amylase and

10 cytokeratin in *Pdx1-Cre* and *Pdx1-Cre;Rb^{ff}* mouse pancreas. Scale bars: 100 μ m. C,

11 Percentage of Ki67-positive acinar cells in *Pdx1-Cre* and *Pdx1-Cre;Rb^{ff}* mice at 2, 4, 6,

12 and 8 months of age (N=3–4). D, Percentage of Ki67-positive duct cells in *Pdx1-Cre* and

13 *Pdx1-Cre;Rb^{ff}* mice at 2, 4, 6, and 8 months of age (N=3–4). E, Body weight of *Pdx1-*

14 *Cre* and *Pdx1-Cre;Rb^{ff}* mice at 1–8 months of age (N=10–21). F, Pancreas weight per

15 body weight of *Pdx1-Cre* and *Pdx1-Cre;Rb^{ff}* mice at 2, 4, 6, and 8 months of age (N=4–

16 5). G, Immunofluorescence staining for synaptophysin, somatostatin, and PP in *Pdx1-Cre*

17 and *Pdx1-Cre;Rb^{ff}* islet cells at 4 months of age. Scale bars: 100 μ m. H, δ -cells per islet

18 cells in *Pdx1-Cre* and *Pdx1-Cre;Rb^{ff}* mice at 2, 4, 6, and 8 months of age (N=4). I, PP-

cells per islet cells in *Pdx1-Cre* and *Pdx1-Cre;Rb^{fl}* mice at 2, 4, 6, and 8 months of age (N=4–6).

Supplemental Figure 2.

A, Experimental strategy for generation of *Pdx1-Cre;Trp53^{R172H}* mice. Arrow indicates the direction of transcription, and arrowheads indicate *loxP* sites. B, H&E, immunohistochemical and immunofluorescence staining of islet cells in *Pdx1-Cre* and *Pdx1-Cre;Trp53^{R172H}* mice at 2 months of age. Arrowheads indicate Ki67-positive cells. Scale bars: 100 μ m. C, α -cell/ β -cell ratio in islet cells in *Pdx1-Cre* and *Pdx1-Cre;Trp53^{R172H}* mice at 2, 4, 6, and 8 months of age (N=3–5). D, Ki67-positive islet cells in *Pdx1-Cre* and *Pdx1-Cre;Trp53^{R172H}* mice at 2, 4, 6, and 8 months of age (N=3–5). E, δ -cells per islet cells in *Pdx1-Cre* and *Pdx1-Cre;Trp53^{R172H}* mice at 2, 4, 6, and 8 months of age (N=4). F, PP-cells per islet cells in *Pdx1-Cre* and *Pdx1-Cre;Trp53^{R172H}* mice at 2, 4, 6, and 8 months of age (N=4–6). G, Blood glucose levels after 4 h fasting in *Pdx1-Cre* and *Pdx1-Cre;Trp53^{R172H}* mice at 2, 4, 6, and 8 months of age (N=7–14). H, Body weight of *Pdx1-Cre* and *Pdx1-Cre;Trp53^{R172H}* mice at 1–8 months of age (N=6–17). I, Pancreas weight per body weight of *Pdx1-Cre* and *Pdx1-Cre;Trp53^{R172H}* mice at 2, 4, 6, and 8 months of age (N=3–4).

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38 **Supplemental Figure 3.**

39 A, H&E, immunohistochemical and immunofluorescence staining of islet cells in *Pdx1-*
40 *Cre* and *Pdx1-Cre;Trp53^{R172H};Rb^{ff}* mice at 2 months of age. Arrowheads indicate Ki67-
41 positive cells. Scale bars: 100 μ m. B, The α -cell/ β -cell ratio in islet cells in *Pdx1-Cre* and
42 *Pdx1-Cre;Trp53^{R172H};Rb^{ff}* mice at 2 and 4 months of age (N=3–5). C, Ki67-positive islet
43 cells in *Pdx1-Cre* and *Pdx1-Cre;Trp53^{R172H};Rb^{ff}* mice at 2 and 4 months of age (N=3–5).
44 D, H&E and immunohistochemical staining for Ki67 of islet cells in *Pdx1-Cre* and *Pdx1-*
45 *Cre;Trp53^{R172H};Rb^{ff}* mice at 2 months of age. Scale bars: 100 μ m. E and F, Representative
46 microscopic images of PanNETs in *Pdx1-Cre;Trp53^{R172H};Rb^{ff}* that have invasiveness to
47 surrounding tissues. Scale bars: 100 μ m. G, Body weight of *Pdx1-Cre* and *Pdx1-*
48 *Cre;Trp53^{R172H};Rb^{ff}* mice at 1–8 months of age (N=6–21). H, Pancreas weight per body
49 weight of *Pdx1-Cre* and *Pdx1-Cre;Trp53^{R172H};Rb^{ff}* mice at 2, 4, 6, and 8 months of age
50 (N=5–6).

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