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Notch1 functions as a tumor suppressor in a model of K-ras-induced pancreatic ductal adenocarcinoma

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Abstract

K-ras is the most commonly mutated oncogene in pancreatic cancer and its activation in murine models is sufficient to recapitulate the spectrum of lesions seen in human pancreatic ductal adenocarcinoma (PDAC). Recent studies suggest that Notch receptor signaling becomes reactivated in a subset of PDACs, leading to the hypothesis that Notch1 functions as an oncogene in this setting. To determine whether Notch1 is required for K-ras-induced tumorigenesis, we employed a mouse model in which an oncogenic allele of *K-ras* is activated and *Notch1* is deleted simultaneously in the pancreas. Unexpectedly, the loss of *Notch1* in this model resulted in increased tumor incidence and progression implying that *Notch1* can function as a tumor suppressor gene in PDAC.

Keywords

Ras; Notch1; Pancreatic Adenocarcinoma; tumor suppressor

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is one of the most aggressive forms of human cancer. Pathogenesis of PDAC is thought to evolve through progression of precursor lesions, termed pancreatic intraepithelial neoplasias (PanINs). The PanINs are classified into four subgroups (1A, 1B, 2, and 3) and eventually evolve into invasive carcinoma. The most commonly mutated oncogene in PDAC is *K-ras*, with activating mutations found in more than 90% of human cases. Recently developed animal models have further underscored this point, as expression of a mutant activated *K-ras* allele in the pancreas is sufficient to induce the

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Author Contributions

L.H., J.L.A., R.M.D., S.T., M.A. and F.R. performed experiments. V.A., F.L. and L.H. analyzed and classified pancreatic pathology. L.H., J.L.A., R.M.D., A.K.R., B.Z.S., F.R., A.J.C. and J.L.K. designed experiments and L.H., J.L.A. and J.L.K. wrote the manuscript. J.L.K. supervised the project.

formation of both pre-malignant and malignant lesions in a mouse model, faithfully recapitulating the human disease (1).

Recent studies have implicated *Notch1* as a potential oncogene in PDAC, as Notch targets appear to become reactivated in a subset of PanINs and PDAC (1-3). The Notch proteins are central components of pancreatic development and are required for directing cell fate decisions and proliferation (4,5). While *Notch1* was originally identified as an oncogene, recent evidence indicates that *Notch1* can also function as a tumor suppressor (6). Conclusive evidence comes from studies in the skin, where loss of both *Notch1* alleles led to development of basal cell carcinoma (7) by mechanisms impacting the tumor microenvironment (8).

Notch and Ras have been shown to cooperate or antagonize one another in a manner that is dependent upon cellular context (9). Previous studies have suggested that the ability of Ras to transform cells depends upon Notch function (10,11). In the case of PDAC, it has been recently shown that ectopic expression of activated Notch1 and K-ras in the mouse pancreas synergizes in inducing PanIN formation (12). Thus, we hypothesized that K-ras and Notch1 functions intersect specifically in the pathogenesis of PDAC. To test this directly, *in vivo*, we generated a compound mutant mouse where *K-ras* is activated and *Notch1* deleted simultaneously in the pancreas. Surprisingly, we found that this resulted in increased tumor incidence and progression implying that *Notch1* can function as a tumor suppressor in a mouse model of PDAC.

Materials and Methods

Mouse strains

The *LSL-Kras^{G12D}* (13), *Notch1^{lox/lox}* (14) and *PDX-1-Cre* (1) mice have been previously described.

Kras^{G12D} and *Notch1* allele recombination PCR assays

Kras^{G12D} allele was analyzed by PCR as described (13). *Notch1* allele recombination PCR assay was performed as described, without multiplexing (15).

Histology and immunohistochemistry

Formalin-fixed paraffin embedded murine pancreatic tissue was processed by standard methods, or subject to immunohistochemical staining, using citrate buffer antigen retrieval. Antibodies: Rat anti-Ki67, (1:400, Dako); rabbit anti-cleaved caspase-3 (1:200, Cell Signaling); rabbit anti-Hey1, (1:125, Abcam); rabbit anti-Hes1 (1:500, B. Stanger, U. Pennsylvania), mouse anti- β -catenin (1:200, BD Biosciences).

Quantitative PCR

Pancreatic tissue samples were snap-frozen. Total RNA was isolated using the Nucleospin RNA II kit (Macherey-Nagel) and reverse transcribed using Superscript II Reverse Transcriptase (Invitrogen). cDNA transcripts were amplified by quantitative real-time PCR using SYBR Green (Applied Biosystems). Detection/quantitation done on ABI Prism 7000 (Applied Biosystems). Each gene was normalized to 18S rRNA. *Notch1* primer sequences: Fwd-TGGATGTCAATGTTTCGAGGA, Rev-CACTGCAGGAGGCAATCAT.

Western blot analysis

Tissues/cells were homogenized in RIPA buffer. Primary antibodies: rabbit anti-Notch1 (1:500, Epitomics), rabbit anti-Notch2, 3 and 4 (all 1:200, Santa Cruz), mouse anti- β -catenin

(1:1000, BD Biosciences), mouse anti-active β -catenin (1:1000, Millipore), rabbit anti-GAPDH (1:10,000, Sigma) and mouse anti-tubulin (1:8000, Sigma).

Isolation and culture of primary pancreatic ductal cells

Primary pancreatic ductal cells (PDCs) were derived as previously described (16). For siRNA experiments, *PDX-1-Cre:Kras^{G12D}:Notch1^{lox/lox}* PDCs were plated at 1×10^4 cells/well in a 12 well plate on day -1, transfected on day 0 with either β -catenin ON-TARGETplus SMART pool siRNA or ON-TARGETplus Non-targeting siRNA pool#3 (Dharmacon), using DharmaFect 2 transfection reagent (Dharmacon) following the manufacturers protocol.

Statistical Analysis

Ki67 positive nuclei comparisons were assessed by a standard unpaired *t*-test. Error bars represent the standard deviation of triplicate counts (p value ≤ 0.05 was considered significant). For real time PCR, mean \pm standard deviation are shown.

Results and discussion

Loss of Notch1 in the context of activated K-ras leads to increased PanIN incidence and progression

To test whether *Notch1* is required for K-ras-induced pancreatic tumorigenesis *in vivo*, we employed a mouse model of PDAC (1). The simultaneous expression of an oncogenic *K-ras* allele and deletion of both *Notch1* alleles was achieved by interbreeding mice harboring both a conditional activated *K-ras* allele (*LSL-K-ras^{G12D}*) (13) and conditional *Notch1^{lox/lox}* knockout alleles (14) with *PDX-1-Cre* transgenic mice that express Cre-recombinase as early as day 8.5 of embryonic development in progenitors of all major pancreatic cell types (17).

We compared the pancreata of *Pdx1-Cre:LSL-K-ras^{G12D}:Notch1^{lox/lox}* mice to those of *Pdx1-Cre:LSL-K-ras^{G12D}* mice. At a 20-week time point, the *PDX-1-Cre:LSL-K-ras^{G12D}* pancreata displayed predominantly a combination of very early changes; acinar to ductal metaplasia (ADM), tubular complexes (TC) and PanIN1A lesions (Fig. 1A). Approximately 50% of the mice displayed ADM/TC, 30% displayed PanIN1A and less than 20% displayed a lesion classified as PanIN1B (Fig. 2A), consistent with previous findings in this model (1). In contrast, 100% of *PDX-1-Cre:LSL-K-ras^{G12D}:Notch1^{lox/lox}* pancreata were classified histologically as PanIN1B, transitioning to PanIN2 (Fig. 1A and 2A). All mice in this cohort displayed tissue architecture that was significantly altered. The majority of ducts exhibited PanIN1B-PanIN2 changes, with only between 10-40% of normal acinar, ductal and islet tissue remaining (Fig. 1B-D and 2A). The appearance of intraepithelial and stromal polymorphonuclear leukocytes (PMNs), a cell type often present in subtypes of human pancreatic cancer and thought to play a role in carcinogenesis, was noted (Fig. 1D). Thus, the *PDX-1-Cre:LSL-K-ras^{G12D}:Notch1^{lox/lox}* mice displayed both increased numbers and more advanced stage PanIN lesions than the *PDX-1-Cre:LSL-K-ras^{G12D}* mice. The *PDX-1-Cre:Notch1^{lox/lox}* pancreata displayed no signs of abnormalities (Fig. 1A and 2A), consistent with recently published findings (18). Likewise, *PDX-1-Cre* pancreata appeared unremarkable (not shown). To verify the expected Cre-mediated recombination events occurred, we isolated genomic DNA from the pancreata and tails of mice from the various genotypes and performed PCR reactions to monitor recombination events. DNA isolated from the pancreas, and not the tails, verified recombination of both alleles (Supp. Fig. 1).

Additionally, mice that are heterozygous for the floxed *Notch1* allele (*Pdx1-Cre:LSL-K-ras^{G12D}:Notch1^{lox/+}*) exhibited an intermediate phenotype. PanIN lesions were predominantly graded as PanIN1B, but a much greater proportion of normal acinar, ductal and islet tissue

remained than when compared to the *PDX-1-Cre:LSL-K-ras^{G12D}:Notch1^{lox/lox}* cohort (not shown).

To compare the proliferative index of the pre-neoplastic lesions in the *PDX-1-Cre:LSL-K-ras^{G12D}* and *PDX-1-Cre:LSL-K-ras^{G12D}:Notch1^{lox/lox}* pancreata, we performed Ki67 immunohistochemical staining and scored the number of positive cells per duct (Fig. 2B). Ki67 positive nuclei were more frequent in similar grade PanINs from *PDX-1-Cre:LSL-K-ras^{G12D}:Notch1^{lox/lox}* pancreata compared to *PDX-1-Cre:LSL-K-ras^{G12D}* pancreata (Fig. 2C). This indicates that in similar grade lesions more cells were actively proliferating in the *PDX-1-Cre:LSL-K-ras^{G12D}:Notch1^{lox/lox}* pancreata. To rule out that decreased rates of apoptosis lead to a more severe phenotype in *PDX-1-Cre:LSL-K-ras^{G12D}:Notch1^{lox/lox}*, we stained sections for cleaved caspase-3. No difference was evident when compared to *PDX-1-Cre:LSL-K-ras^{G12D}* pancreata (Fig. 2D). These studies demonstrate that the loss of Notch1 in the context of activated K-ras results in increased proliferation rates of pancreatic ductal cells *in vivo*, increased PanIN incidence, and progression. This implies that Notch1 possesses tumor suppressor-like function in a mouse model of Kras-induced PDAC.

In a recent report, conditionally co-expressing activated Notch and K-ras in mouse pancreata induced synergy in PanIN formation (12). This was interpreted as an indication of Notch1 functioning to inhibit the normal differentiation of the tumor initiating cells in the pancreas. It is possible that differences in target cells and/or timing of recombination events might account for the differences between our findings. In addition, Notch1 expression and activation are highly regulated and overexpression of a constitutively active form of Notch1 could lead to non-physiological phenomena. Finally, a recent report which catalogued core signaling pathways in human pancreatic cancer suggests that the expression levels of Notch-family members and downstream targets were not up-regulated in primary tumor samples and cell lines, when compared to normal pancreatic ductal epithelium (19).

Activation of Notch family members is not responsible for accelerated tumorigenesis in *PDX-1-Cre:LSL-K-ras^{G12D}:Notch1^{lox/lox}* mice

To assess whether other Notch family members (Notch 2- 4) are induced to compensate and substitute for the loss of Notch1, we examined the expression and activation of the Notch family receptors. Employing quantitative PCR to verify loss of Notch1 expression we find, as expected, that expression of Notch1 mRNA was reduced in both the *PDX-1-Cre:Notch1^{lox/lox}* and *PDX-1-Cre:LSL-K-ras^{G12D}:Notch1^{lox/lox}* pancreata, when compared to control *PDX-1-Cre* pancreata. In the *PDX-1-Cre:LSL-K-ras^{G12D}* pancreata the expression of Notch1 mRNA appeared to be slightly upregulated when compared to *PDX-1-Cre* (Fig. 3A). Additionally, we analyzed Notch1 protein levels by western blotting, confirming loss of Notch1 in *PDX-1-Cre:LSL-K-ras^{G12D}:Notch1^{lox/lox}* and *PDX-1-Cre:Notch1^{lox/lox}* pancreata (Fig. 3B). Western blot analysis of Notch2, 3 and 4, in extracts prepared from pancreas tissue, as above, indicates no significant differences in levels of expression between the cohorts (Fig. 3C). To further establish whether the activity of Notch family members might be increased in the pancreas of *PDX-1-Cre:LSL-K-ras^{G12D}:Notch1^{lox/lox}* mice we analyzed the expression of established Notch downstream target genes, Hes1 and Hey1, by immunohistochemistry. Expression of Hes1 and Hey1 was detected at similar levels in comparable grade lesions of *PDX-1-Cre:LSL-K-ras^{G12D}:Notch1^{lox/lox}* mice compared to *PDX-1-Cre:LSL-K-ras^{G12D}* (Fig. 3D). Collectively our data indicate that *Notch1* has been effectively deleted in the pancreas of *PDX-1-Cre:LSL-K-ras^{G12D}:Notch1^{lox/lox}* mice, and that activation of other Notch family members is not likely to account for the observed acceleration of tumorigenesis in these mice.

Activation of β -catenin in pancreata of *PDX-1-Cre:LSL-K-ras^{G12D}:Notch1^{lox/lox}* mice

To investigate potential mechanisms underlying the observed tumor suppressive function of Notch1 in K-ras-induced PDA, we examined the status of β -catenin, which has been identified as a target of Notch1's tumor suppressor function in the skin (4,7). In normal adult pancreas, localization at the cell membrane serves as an indication of inactivity while both cytoplasmic and nuclear localization of β -catenin are commonly regarded as indicators of active canonical Wnt/ β -catenin signaling (20). In *PDX-1-Cre* acinar and ductal cells, total β -catenin was restricted to the cell membrane (Fig. 4A). In *PDX-1-Cre:LSL-K-ras^{G12D}* β -catenin was observed mostly in ductal cells at the cell membrane and faintly in the cytoplasm (Fig. 4A). In contrast, intense β -catenin staining was observed in the membrane and cytoplasm in *PDX-1-Cre:LSL-K-ras^{G12D}:Notch1^{lox/lox}* ductal cells, implying β -catenin levels are induced and possibly activated (Fig. 4A). Importantly, total β -catenin levels were similar in pancreatic cells of all genotypes (Fig. 4B).

To further assess β -catenin activation directly in pancreatic ductal cells, we isolated primary pancreatic ductal cells (PDCs) from each of the different genotypes. Western blot analysis revealed no increase in either total or activated β -catenin (dephosphorylated on Ser37 and Thr41) in PDCs derived from *PDX-1-Cre:LSL-K-ras^{G12D}:Notch1^{lox/lox}* mice compared to the other genotypes (Fig. 4C). Finally, in order to functionally determine if β -catenin is required for proliferation of *PDX-1-Cre:LSL-K-ras^{G12D}:Notch1^{lox/lox}* PDCs, β -catenin was knocked down in these cells using an siRNA-based approach. Cells treated with β -catenin siRNA displayed a significant reduction in β -catenin levels, however this knockdown did not effect the proliferative capacity of these cells, when compared to untreated cells or cells treated with non-targeting siRNA (Fig. 4D).

These findings suggest that β -catenin repression might not represent the putative tumor suppressive function of Notch1 in our mouse model of K-ras-induced PDAC. This conclusion is based on studies in PDCs and it is possible that these findings do not reflect the complex interactions occurring *in vivo*.

In conclusion, we show that loss of Notch1, in the context of activated K-ras, leads to acceleration of tumor progression and an increase in PanIN numbers in a mouse model of PDAC. This implies that Notch1 can function as a tumor suppressor in K-ras-induced PDAC and additional studies are required to determine which downstream effectors of Notch1 signaling are essential for this activity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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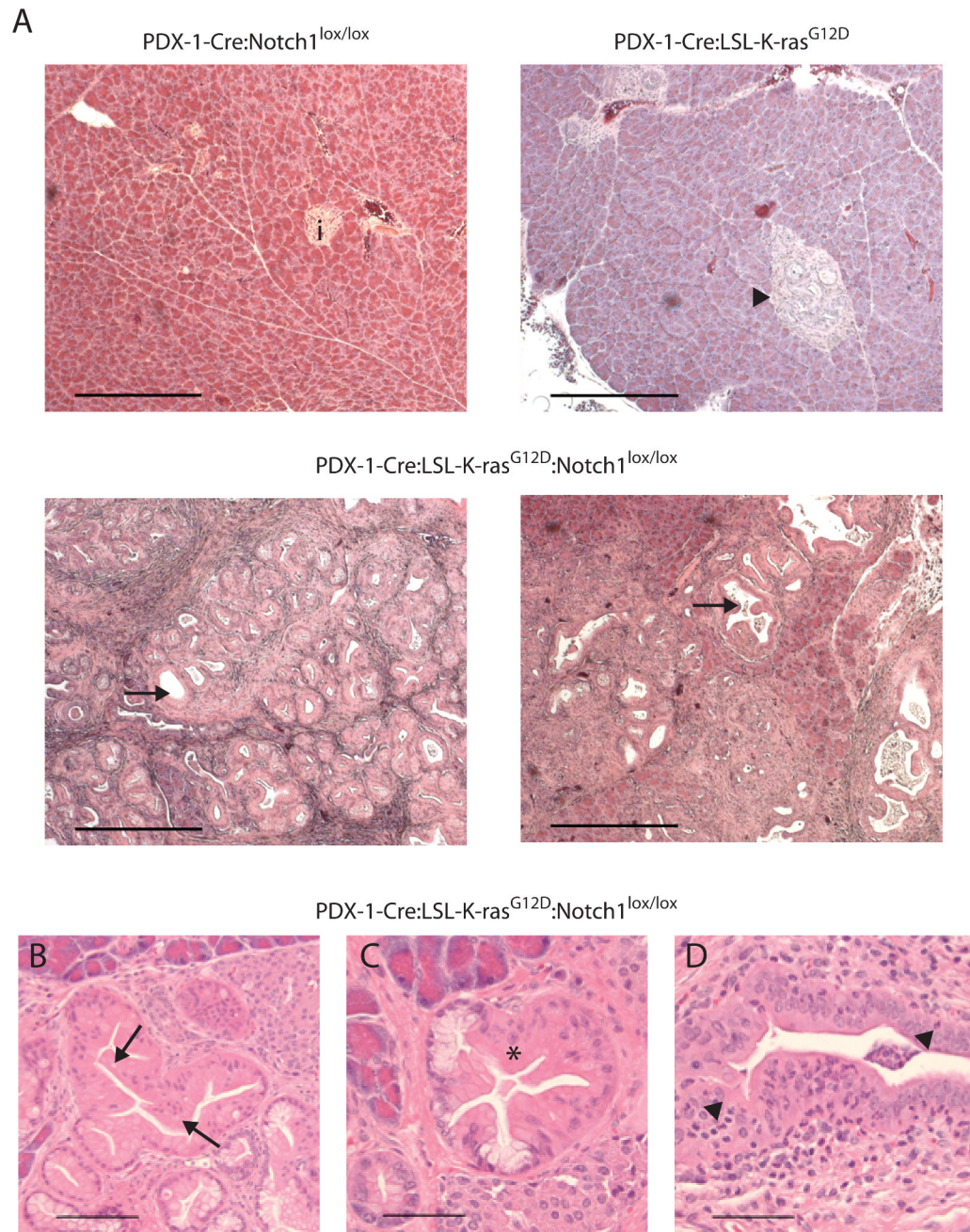


Figure 1. Histological analysis of pancreata

(A) H&E stained sections from pancreata at 20 weeks of *PDX-1-Cre:Notch1^{lox/lox}* mice (i = islet), *PDX-1-Cre:LSL-K-ras^{G12D}* mice (arrowhead = tubular complexes-TC), and *PDX-1-Cre:LSL-K-ras^{G12D}:Notch1^{lox/lox}* mice (arrows = ducts exhibiting PanIN 1B-2 changes). Scale bar = 400 μ m. (B-D) Detailed characterization of pathology exhibited in *PDX-1-Cre:LSL-K-ras^{G12D}:Notch1^{lox/lox}* pancreata. (B) PanIN1B; papillary ductal lesions without significant loss of polarity or nuclear atypia (arrows). (C) PanIN1B transiting to PanIN2 revealing moderate nuclear atypia and a loss of polarity. (D) PanIN1B showing intraepithelial polymorphonuclear leukocytes (arrowheads). Scale bar = 200 μ m.

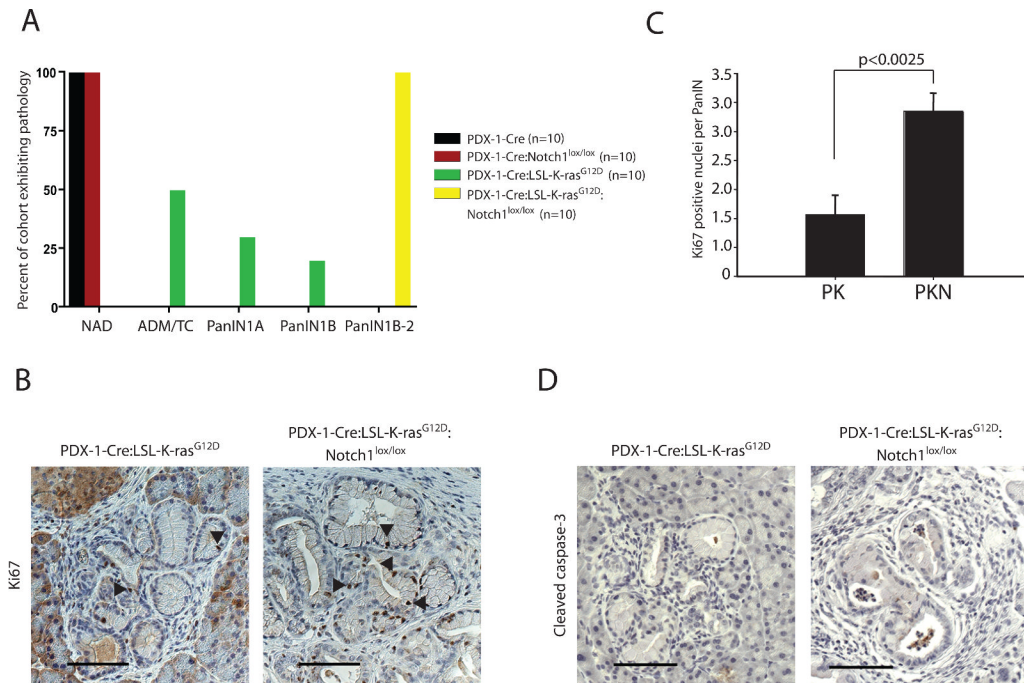


Figure 2. Characterization of pancreatic pathology in the experimental cohorts

(A) Grouped illustration of predominant pancreatic pathology in each of the four experimental cohorts at 20 weeks; NAD – no abnormality detected, ADM/TC – acinar to ductal metaplasia/tubular complexes, PanINs – pancreatic intraepithelial neoplasias. N=10 animals from each cohort (B) Representative Ki67 staining in *PDX-1-Cre:LSL-K-ras^{G12D}* and *PDX-1-Cre:LSL-K-ras^{G12D}:Notch1^{lox/lox}* pancreata. Arrowheads point to Ki67 positive nuclei. Scale bar = 80 μ m. (C) Number of Ki67 positive nuclei per PanIN lesion in pancreata from *PDX-1-Cre:LSL-K-ras^{G12D}* (PK) and *PDX-1-Cre:LSL-K-ras^{G12D}:Notch1^{lox/lox}* (PKN) mice. Six pancreata from each genotype were analyzed; 100 PanINs per pancreata were counted. (D) Representative cleaved caspase-3 staining in *PDX-1-Cre:LSL-K-ras^{G12D}* and *PDX-1-Cre:LSL-K-ras^{G12D}:Notch1^{lox/lox}* pancreata. Scale bar = 80 μ m.

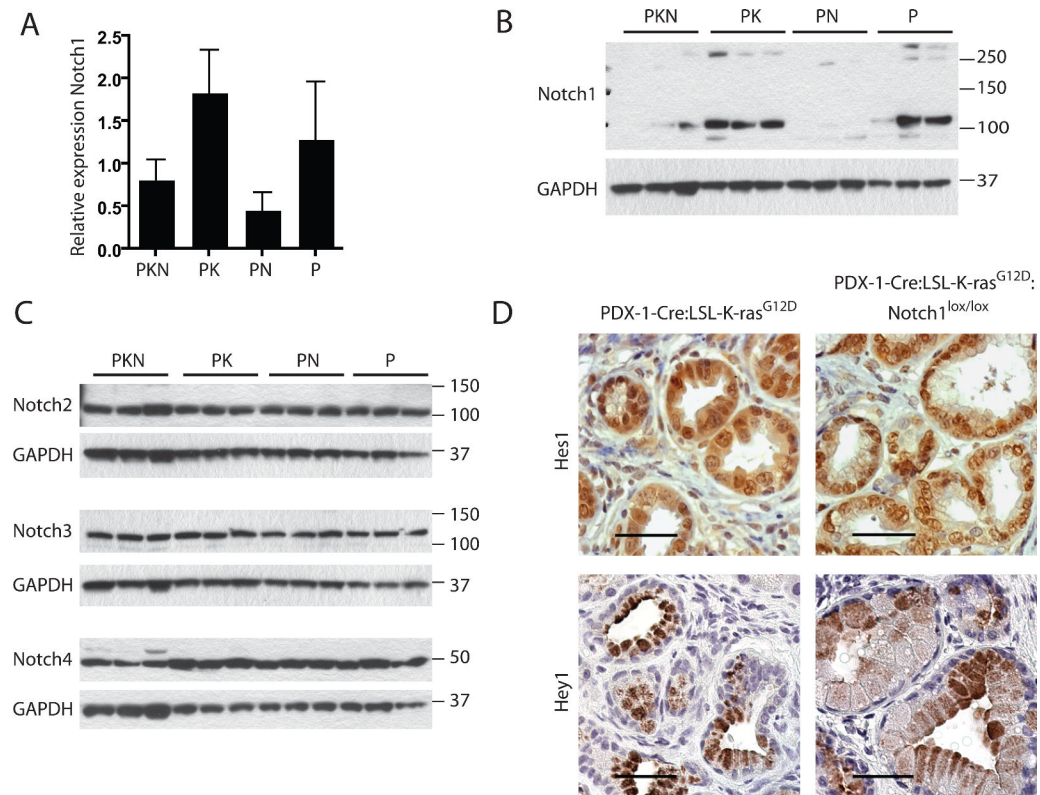


Figure 3. Expression of Notch family members and targets in pancreata from cohorts
(A) Relative expression levels of Notch1 in pancreatic tissue determined by qPCR (normalized to 18srRNA). **(B)** Western blot analysis of Notch1 in whole pancreatic lysates. Both the full length Notch1 (250kDa) and cleaved Notch1 intracellular domain (120kDa) are detected. Three independent samples are shown for each genotype. PKN=*PDX-1-Cre:LSL-K-ras^{G12D}:Notch1^{lox/lox}*, PK=*PDX-1-Cre:LSL-K-ras^{G12D}*, PN=*PDX-1-Cre:Notch1^{lox/lox}*, P=*PDX-1-Cre*. (GAPDH =loading control). **(C)** Expression of Notch 2, 3, and 4 in whole pancreatic lysate determined by western blot analysis. Three independent samples are shown for each genotype. (GAPDH =loading control). **(D)** Immunohistochemical analysis of Hes1 and Hey1 in similar grade PanIN lesions. Scale bar = 40 μ m.

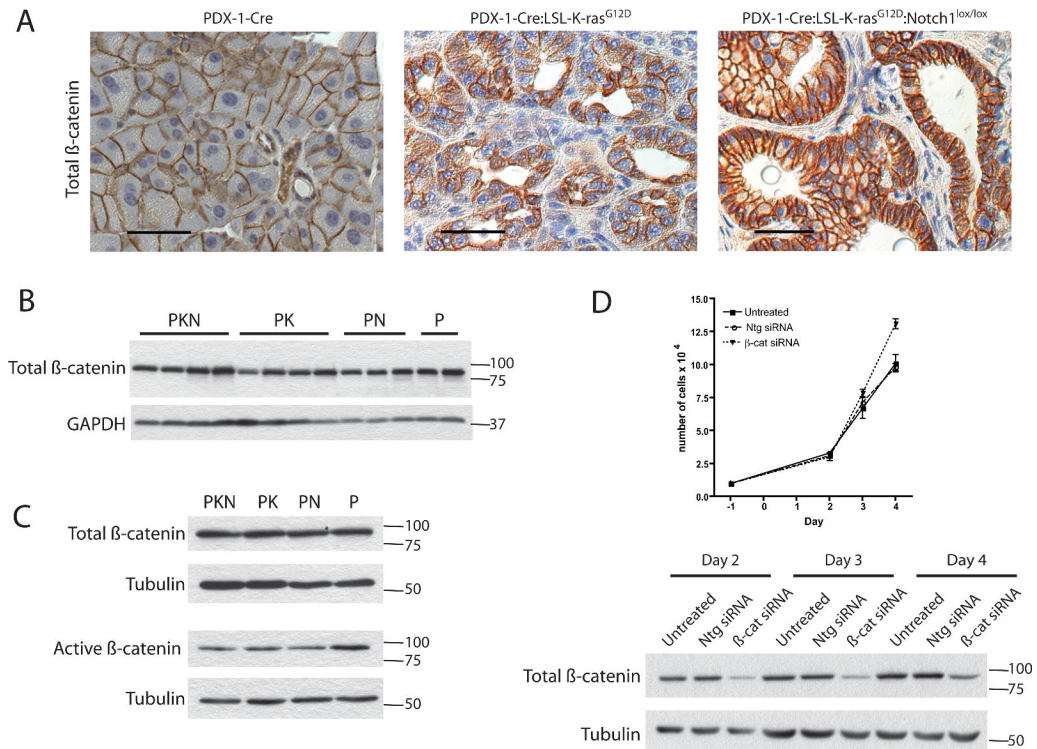


Figure 4. Expression and activation state of β -catenin in pancreata from cohorts

Expression and localization of total β -catenin (A) (Scale bar = 40 μ m) by immunohistochemical staining in pancreata from *PDX-1-Cre*, *PDX-1-Cre:LSL-K-ras^{G12D}*, and *PDX-1-Cre:LSL-K-ras^{G12D}:Notch1^{lox/lox}* mice. (B) Expression levels of total β -catenin in whole pancreatic lysates by western blot analyses. Four independent samples are shown for *PDX-1-Cre:LSL-K-ras^{G12D}:Notch1^{lox/lox}* (PKN) and *PDX-1-Cre:LSL-K-ras^{G12D}* (PK) cells, 3 independent samples for *PDX-1-Cre:Notch1^{lox/lox}* (PN) cells and 2 independent samples for *PDX-1-Cre* (P) cells. (GAPDH = loading control). (C) Western blot analysis of total and activated β -catenin expression in PDCs. (Tubulin = loading control). Results shown are representative of two independent PDC lines tested for each genotype. (D) Proliferation of *PDX-1-Cre:LSL-K-ras^{G12D}:Notch1^{lox/lox}* PDCs untreated, treated with Non-targeting siRNA pool (Ntg siRNA), or β -catenin siRNA pool (β -cat siRNA). Values shown are the mean of 3 independent samples. Western blot analysis of β -catenin knockdown in PDCs treated with siRNA on days 2, 3, and 4. (Tubulin = loading control).