

Priority Report

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 used 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. *Cancer Res*; 70(11): 4280–6. ©2010 AACR.

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

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L. Hanlon and J.L. Avila contributed equally to this work.

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Recent studies have implicated *Notch1* as a potential oncogene in PDAC, as Notch targets seem 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). Although *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 affecting the tumor microenvironment (8).

Notch and Ras have been shown to cooperate or antagonize one another in a manner that is dependent on cellular context (9). Previous studies have suggested that the ability of Ras to transform cells depends on 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* is 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-K-ras^{G12D}* (13), *Notch1^{lox/lox}* (14), and *PDX-1-Cre* (1) mice have been previously described.

K-ras^{G12D} and *Notch1* allele recombination PCR assays

LSL-K-ras^{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 subjected to immunohistochemical staining, using citrate buffer antigen retrieval. Antibodies were 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, University of Pennsylvania, Philadelphia, PA), and mouse anti- β -catenin (1:200; BD Biosciences).

Quantitative PCR

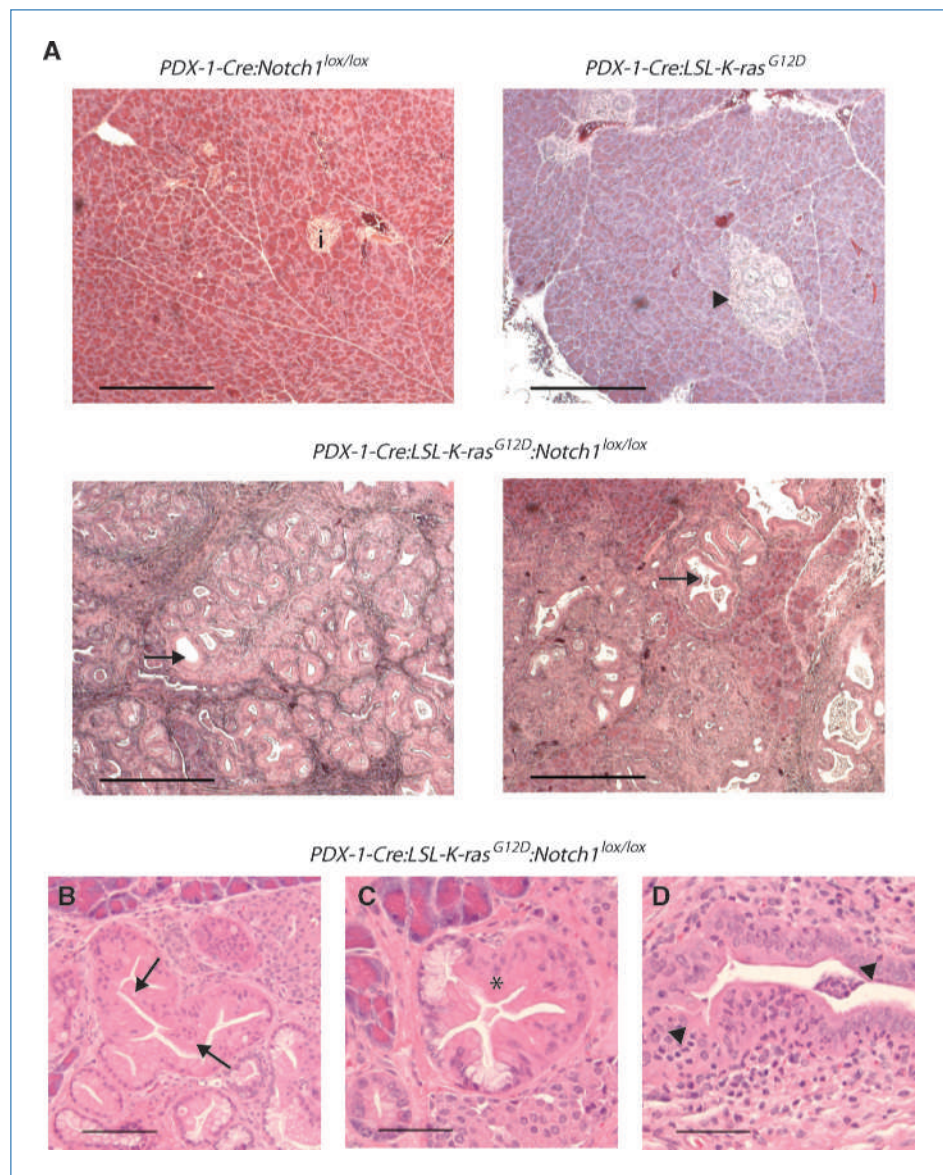
Pancreatic tissue samples were snap-frozen. Total RNA was isolated using the Nucleospin RNA II kit (Macherey-Nagel) and reverse transcribed using Superscript II Reverse Transcriptase (Invitrogen). cDNA transcripts were amplified

by quantitative real-time PCR using SYBR Green (Applied Biosystems). Detection/quantitation was done on ABI Prism 7000 (Applied Biosystems). Each gene was normalized to 18S rRNA. Notch1 primer sequences were fwd-TGGATGT-CAATGTTTCGAGGA and rev-CACTGCAGGAGGCAATCAT.

Western blot analysis

Tissues or cells were homogenized in radioimmuno-precipitation assay buffer. Primary antibodies were rabbit anti-Notch1 (1:500; Epitomics), rabbit anti-Notch2, anti-Notch3, and anti-Notch4 (all 1:200; Santa Cruz), mouse anti- β -catenin (1:1,000; BD Biosciences), mouse anti-active β -catenin (1:1,000; Millipore), rabbit anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 1:10,000; Sigma), and mouse anti-tubulin (1:8,000; Sigma).

Figure 1. Histologic analysis of pancreata. A, H&E-stained sections of pancreata at 20 wk from *PDX-1-Cre:Notch1^{lox/lox}* mice (i, islet), *PDX-1-Cre:LSL-K-ras^{G12D}* mice (arrowhead, tubular complexes), and *PDX-1-Cre:LSL-K-ras^{G12D}; Notch1^{lox/lox}* mice (arrows, ducts exhibiting PanIN 1B-2 changes). Bar, 400 μ m. B to 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 transitioning to PanIN2, revealing moderate nuclear atypia and a loss of polarity. D, PanIN1B showing intraepithelial polymorphonuclear leukocytes (arrowheads). Bar, 200 μ m.



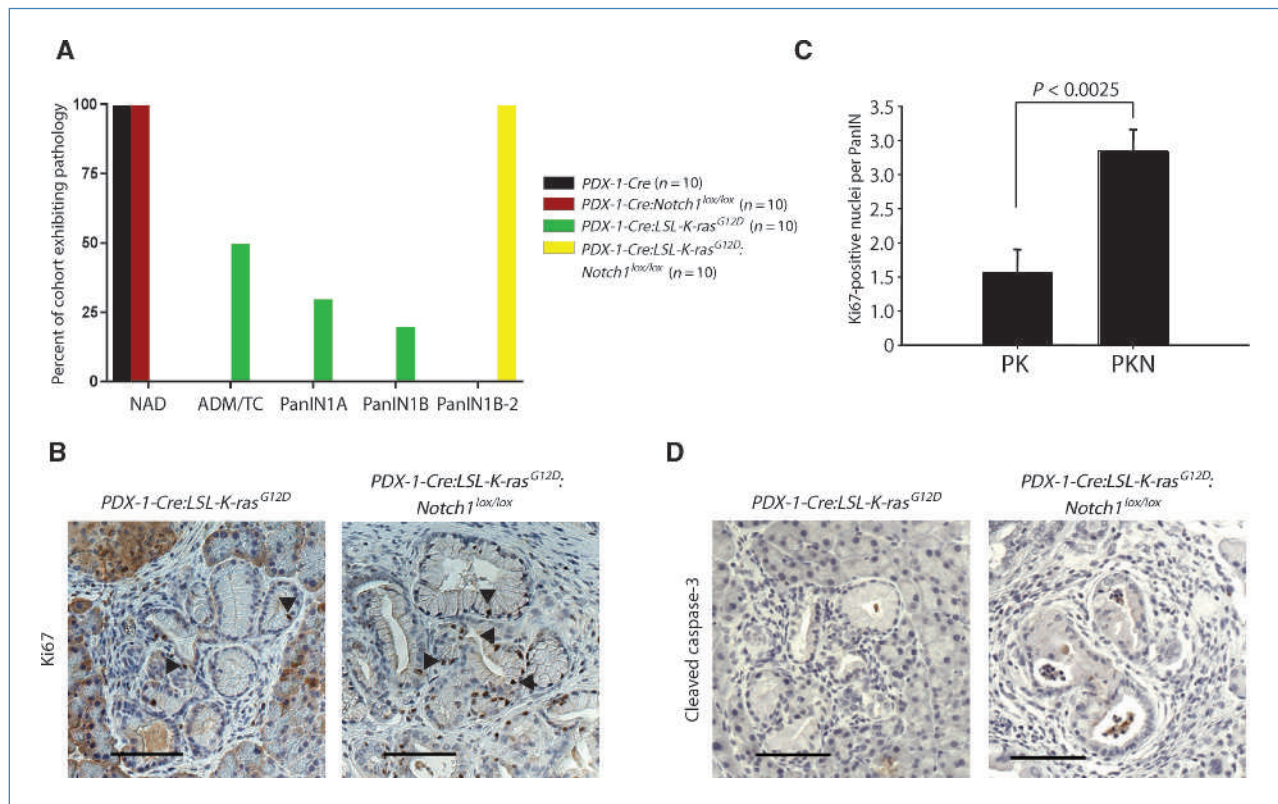


Figure 2. Characterization of pancreatic pathology in the experimental cohorts. A, grouped illustration of predominant pancreatic pathology in each of the four experimental cohorts at 20 wk. NAD, no abnormality detected; ADM/TC, acinar to ductal metaplasia/tubular complexes. *n* = 10 animals from each 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, Ki67-positive nuclei. 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. Bar, 80 μ m.

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:K-ras^{G12D}; Notch1^{lox/lox}* PDCs were plated at 1×10^4 per well in a 12-well plate on day -1, then transfected on day 0 with either β -catenin ON-TARGETplus SMART pool siRNA or ON-TARGETplus Nontargeting 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 SD of triplicate counts (*P* < 0.05 was considered significant). For real-time PCR, mean \pm SD 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 used a mouse model of PDAC (1). The simultaneous expression of an oncogenic

K-ras allele and the deletion of both *Notch1* alleles was achieved by interbreeding mice harboring both a conditional activated *K-ras* allele (*LSL-K-ras^{G12D}*; ref. 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 *PDX-1-Cre:LSL-K-ras^{G12D}; Notch1^{lox/lox}* mice to those of *PDX-1-Cre:LSL-K-ras^{G12D}* mice. At the 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 (Figs. 1A and 2A). All mice in this cohort displayed a tissue architecture that was significantly altered. The majority of ducts exhibited PanIN1B-PanIN2 changes, with only between 10% and 40% of normal acinar, ductal, and islet tissue remaining (Figs. 1B–D and 2A). The

appearance of intraepithelial and stromal polymorphonuclear leukocytes, 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 (Figs. 1A and 2A), consistent with recently published findings (18). Likewise, *PDX-1-Cre* pancreata appeared unremarkable (not shown). To verify whether 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 (Supplementary Fig. S1).

Additionally, mice that are heterozygous for the floxed *Notch1* allele (*PDX-1-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 with the *PDX-1-Cre:LSL-K-ras^{G12D}:Notch1^{lox/lox}* cohort (not shown).

To compare the proliferative index of the preneoplastic 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 with *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

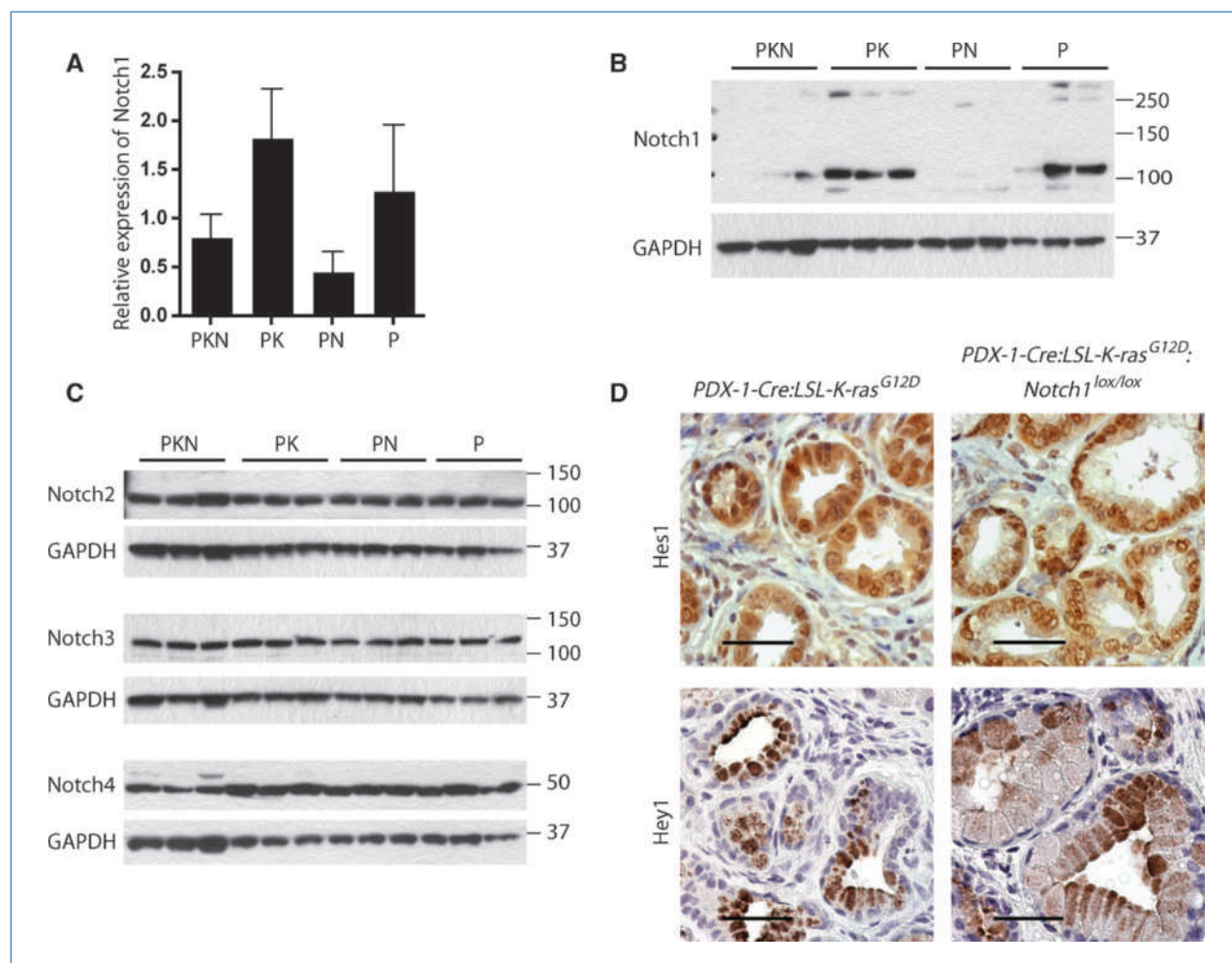


Figure 3. Expression of Notch family members and targets in pancreata from cohorts. A, relative expression levels of Notch1 in pancreatic tissue determined by quantitative PCR (normalized to 18S rRNA). B, Western blot analysis of Notch1 in whole pancreatic lysates. Both full-length Notch1 (250 kDa) and cleaved Notch1 intracellular domain (120 kDa) 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 Notch2, Notch3, and Notch4 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. Bar, 40 μ m.

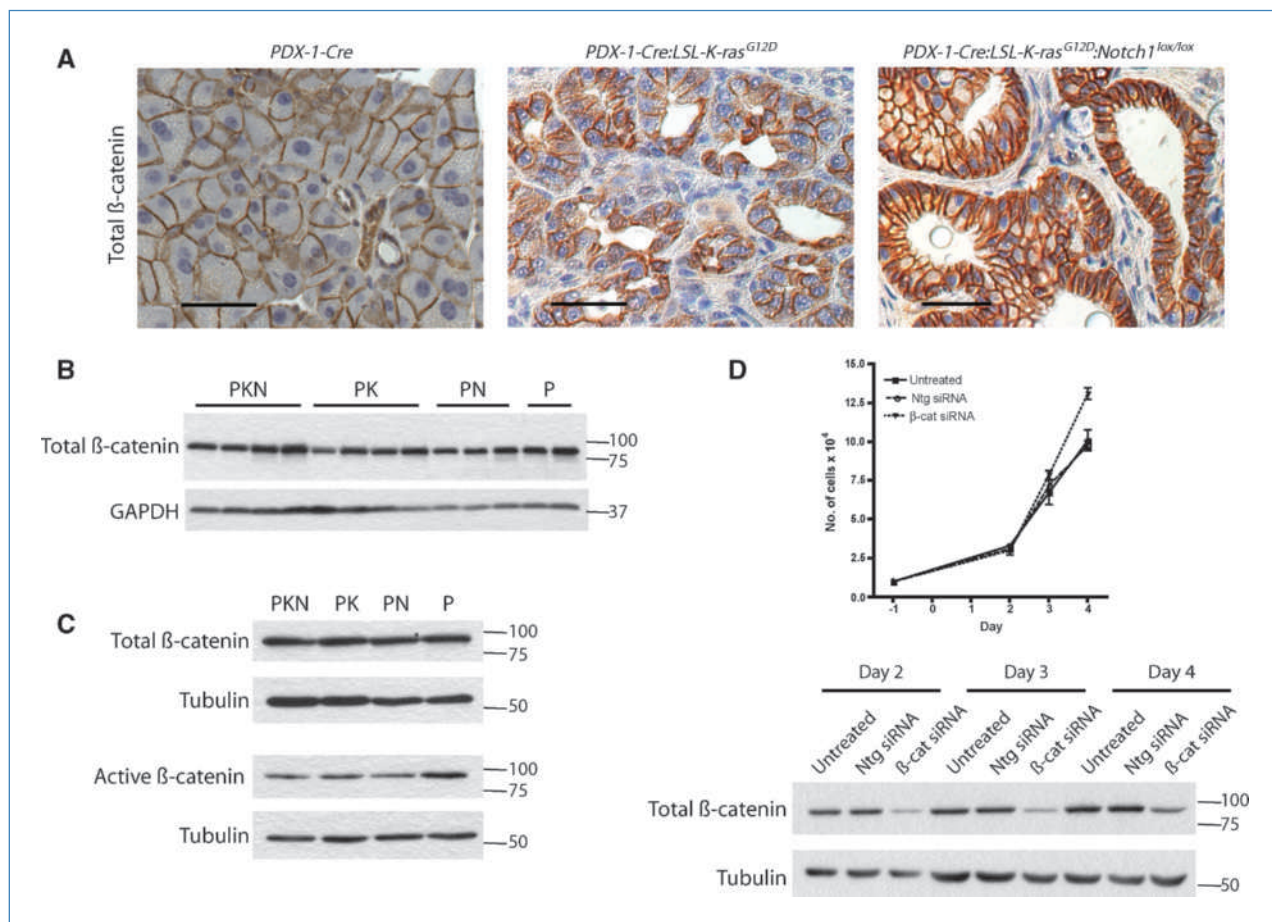


Figure 4. Expression and activation state of β -catenin in pancreata from cohorts. Expression and localization of total β -catenin (A; bar, 40 μ m) by immunohistochemical staining in pancreata from *PDX-1-Cre*, *PDX-1-Cre:LSL-K-ras^{G12D}* and *PDX-1-Cre:LSL-K-ras^{G12D};Notch1^{lox/lox}* mice. B, expression 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, three independent samples for *PDX-1-Cre:Notch1^{lox/lox}* (PN) cells, and two 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 or treated with nontargeting siRNA pool (Ntg siRNA) or β -catenin siRNA pool (β -cat siRNA). Values shown are the mean of three independent samples. Western blot analysis of β -catenin knockdown in PDCs treated with siRNA on days 2, 3, and 4. Tubulin, loading control.

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 with *PDX-1-Cre:LSL-K-ras^{G12D}* pancreata (Fig. 2D). These studies show 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 K-ras-induced PDAC.

In a recent report, conditionally coexpressing 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 nonphysiologic 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 upregulated in primary tumor samples and cell lines, when compared with 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 (Notch2–Notch4) 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 the *PDX-1-Cre:LSL-K-ras^{G12D};Notch1^{lox/lox}* pancreata, when compared with control *PDX-1-Cre*

pancreata. In the *PDX-1-Cre:LSL-K-ras^{G12D}* pancreata, the expression of Notch1 mRNA seemed to be slightly upregulated when compared with *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, Notch3, and Notch4 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 pancreata 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 with *PDX-1-Cre:LSL-K-ras^{G12D}* (Fig. 3D). Collectively, our data indicate that *Notch1* has been effectively deleted in the pancreata 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 the Notch1 tumor suppressor function in the skin (4, 7). In normal adult pancreas, localization at the cell membrane serves as an indication of inactivity, whereas 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 that 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 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 with the other genotypes (Fig. 4C). Finally, 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 affect the proliferative capacity of these cells when compared with untreated cells or cells treated with nontargeting 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.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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