Supplementary Materials and Methods

Genetically Engineered Mice

Conditional $Rb^{L/L}$ and $LSL\text{-}Kras^{G12D}$ mice were obtained from the Mouse Models of Human Cancer Consortium at NCI (strain numbers 01XC1 and 01XJ6, respectively). *Pdx1-Cre* mice were kindly provided by Dr. G.Gu (Vanderbilt University Medical Center, Nashville, TN). The triple mutant $Rb^{L/L}$*; Pdx1-Cre ;*$LSL\text{-}Kras^{G12D}$ mice, and various littermate controls were on a mixed FVB; 129; C57Bl/6 genetic background.

Histology, Immunohistochemistry and Immunofluorescence:

Mice were perfused with PBS then 10% formalin (2 min each). Tissues were dissected, fixed in 10% formalin overnight, embedded in paraffin and 4-5 μm sections were prepared. Immunostaining procedures were performed as described.[1] If required, antigen unmasking solution (Vector Labs, Burlingame, CA) was used according to manufacturer instructions. A Proteinase K procedure was used for CK19 staining. The antibodies and dilutions used were: CK19/TromaIII (1:10, contributed by Dr. R. Kremler to Developmental Studies Hybridoma Bank, Iowa City, IA;); Hes1 (1:400; a gift from Dr. T. Sudo, Toray Inc., Kamakura, Japan); β-Catenin (1:500; R&D Systems, Minneapolis, MN); Mac3 (1:50; BD Pharmingen, San Diego, CA); PR (SP2) (1:100; Thermo, San Diego, CA); Ki67 (1:200) and p53 (1:500 (Novocastra, UK); MPO (1:300) and ER (1D5) (1:100) were from Dako (Denmark); γ-H2AX (Ser139) (1:200), and cleaved caspase 3 (Asp175) (1:200) were from Cell Signaling Technology, Inc. (Danvers, MA); Pax5 (C-20), CD3ε (M-20), $p21^{Cip1/Waf1}$ (F-5) and $p16^{Ink4a}$ (M-156) (all 1:50; Santa Cruz Biotechnology, Santa Cruz, CA); amylase (Calbiochem, 1:500); insulin (Dako, 1:500); Glucagon (ab10988, 1:500; Abcam, Cambridge, MA); α-tubulin (DM1A) (1:500) and γ-tubulin (T3559, 1:5,000) (Sigma-Aldrich, St. Louis, MO). Immunohistochemical detection was done using matched, biotinylated secondary antibodies, and ABC Elite and NOVA RED kits (Vector Labs). Immunofluorescence on cells was done following a 15 minute fixation in 10% formalin. Secondary antibodies for immunofluorescence were from Molecular Probes (Carlsbad, CA).

Alcian blue staining was done as described.[1] For Masson's trichrome staining, a Chromaview kit (Thermo, Waltham, MA) was used according to manufacturer instructions.

SA-LacZ staining was done using 10 μm cryosections as described in.[1] Briefly, tissues were fixed in 10% formalin for 15 min, washed in PBS and transferred to PBS/30% sucrose. Following overnight storage at 4 C, tissues were frozen in OCT compound (Sakura Finetek, Torrance, CA). When accompanied by immunohistochemistry, antigen retrieval was done after SA-LacZ staining. Samples were analyzed on an Olympus BX60, and pictures were taken with an Olympus DP70 or QImaging EXI Blue cameras using ImagePro for imaging and counting.

Histopathological Evaluation

H&E stained sections were evaluated by a pathologist with specific expertise in human pancreatic cancer (DSL). The analysis was done blind without knowledge of the genotypes of the specimen and the pathways mutated.

Immunoblots

Protein lysates were prepared and immunoblot experiments performed as previously described.[2] To induce DNA damage, cells were subjected to 10 Gy of γ-irradiation (10 Gy/min) using a model 81-14R cesium irradiator (J.L. Shepherd and Associates, San Fernando, CA), and lysates were obtained 1 h post-irradiation. Antibodies and dilutions used include: p21 (F-5) and p-ATM (Ser1981) (1:250), and ERK2 (C-14, 1:10,000) were from Santa Cruz Biotechnology; RB (D20), p-p53 (Ser18), ATM, p-CHK1 (Ser345), CHK1 and γ-H2AX (all 1:1,000; Cell Signaling Technology, Inc); MDM2 (ab38619) (1:100) and p19 (Ab80) (1:1,000) were from Abcam. HRP-conjugated secondary antibodies were from Bio-Rad.

Primary Pancreatic Cell Lines and Mutation Analyses of p53

Primary cell lines were prepared as previously described.[3] All *in vitro* analyses were performed on cells that had been passaged <12 times, with passage one starting when epithelial cell number reached 80%. RNA was extracted from cell lines using the RNeasy mini kit (Qiagen, Valencia, CA) and cDNA was prepared using the Superscript III First Strand cDNA Synthesis Kit (Invitrogen, Carlsbad, CA) with oligodT primers following the manufacturers' recommendations. PCR using PfuUltra HF DNA polymerase (Stratagene, La Jolla, CA) was

performed to amplify p53 cDNA using the primers below. The PCR products were directly sequenced on both strands using the same primers used for PCR amplification.

p53 primer sequences:

5'-GTCTGAATTCACTGGATGACTGCCATGGAGG-3'

5'-CGTTGGATCCAGGCAGTCAGTCTGAGTCAGG-3'

_Rb_ Recombination

Genomic DNA was isolated using the All Prep DNA kit (Qiagen) and recombination of *Rb* was assessed as described in.[4] To show that exon19 was deleted in Rb/K cell lines, RT-PCR was performed using the primers below. These primers flank exon19, resulting in a 150 bp smaller PCR product following *Rb* recombination.

Rbe18s: 5'-AAGCAGTCCAAGGATGGAGAAGGA-3'

Rbe19as: 5'-ACACGTTTAAAGGTCTCCTGGGCA-3'

Proliferation Assays

To monitor cell growth, we used the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide growth assay as described in.[5] Colorimetric changes were measured 72 h after TGF-β1 treatment using a microtiter plate reader with a 570 nm filter. Data for control-treated cells were normalized to 100%, and changes in growth were calculated as percent of control. Data were plotted as the mean percentage +/- SEM from three independent experiment (*$P < .05$).

Quantitative RT- PCR

To assess gene expression levels, cDNA was prepared from cell line and pancreatic RNA using the Superscript III First Strand cDNA Synthesis Kit and oligo dT primers (Invitrogen). Taqman reactions (25 μl) were set up using 2× Universal PCR Master Mix (Applied Biosystems, Foster City, CA), pre-validated primer and probe sets (Assays on Demand, Applied Biosystems), and equivalent amounts of template cDNA. qPCR was performed with an ABI PRISM 7300 sequence detection system (Applied Biosystems). Each sample was assayed in duplicate, and

Rps6 served as the endogenous control for normalization.[6] The expression levels for each gene were calculated relative to the level in control cells or wild-type littermate control pancreata using the $2^{-\Delta\Delta Ct}$ method.[7] Data obtained from cell lines were plotted as the mean +/- SEM from three independent experiments. For *Rb/K* animals, 6 different pancreata were used, and for littermate controls (Ct(*Rb*)), 9 animals were used; 3 animals each of the following genotypes: $Rb^{L/L}$, *Pdx1-Cre*; $Rb^{L/L}$ and $Kras^{G12D}$; $Rb^{L/L}$. For *K* mice, three 3 month-old, and four 6 month-old pancreata were used. Three littermate control mice (Ct(*K*)) were used for each age group, including wild-type, *Pdx1-Cre* without *Kras* and $Kras^{G12D}$ without *Pdx1-Cre*. Data were presented as the relative level of expression in each animal.

Luciferase Assays

p53 transcriptional activity was assayed using two different p53 responsive reporters: $(p53RE)_5$-tk-luc (a kind gift from Dr. James Direnzo, Dartmouth Medical School, Hanover, NH) and p53-TA-Luc (Clontech, Mountain View, CA). Briefly, cells were plated in triplicate in a 6-well format and transfected with $(p53RE)_5$-tk-luc or p53-TA-Luc using Lipofectamine 2000 (Invitrogen). pCMV-β-gal (Clontech) was co-transfected to control for transfection and lysis efficiency. Following 24 h of transfection, cells were γ-irradiated (10 Gy). After 1 h, cells were lysed in 1x Passive Lysis Buffer (Promega, Madison, WI) and luciferase assays were performed using the Dual Luciferase Assay Kit (Promega) and a LMaxII microplate reader (Molecular Devices, Sunnyvale, CA) following manufacturers' recommendations. In parallel, β-galactosidase activity was determined for each extract using a standard *o*-nitrophenyl-β-D-galactopyranoside (ONPG) assay. Colorimetric changes were measured with a microtiter plate reader and a 405 nm filter. Data from three independent experiments were presented as the mean ratio of Luciferase/β-galactosidase activity +/- SEM for irradiated or control-treated cells. Shown in Figure 6B are results obtained using the $(p53RE)_5$-tk-luc reporter. Similar results were obtained with the p53-TA-Luc reporter (not shown). A one-way ANOVA was used for statistical analysis ($*P < .01$).

Spectral Karyotyping (SKY)

Chromosome analysis on K (K1 and K2) and Rb/K (Rb/K338, 343-1, -5 and -6) cells was performed by the Cancer Cytogenetics Core at Texas Children's Hospital (Houston, TX) as

described.[8] Briefly, hybridization and detection of mouse chromosome paints (ASI, Vista, CA) was performed according to the manufacturer's protocol, and chromosomes were counterstained with DAPI. For each cell line, a minimum of 10 metaphase cells was analyzed. Images were acquired with a SD300H Spectra cube (ASI) mounted on a Zeiss Axioplan II microscope using a custom designed optical filter (SKY-1) (Chroma Technology, Brattleboro, VT), and analyzed using SKY View 2.1.1 software (ASI, Vista, CA).

Microarrays

Total RNA from K (K1 and K2) and Rb/K (Rb/K338, 343-1 and 343-6) cell lines was extracted using Trizol (Invitrogen). Gene expression analysis and bioinformatics was performed by Miltenyi Biotec (Bergisch Gladbach, Germany) using two-color Agilent whole mouse genome arrays, and pooled K (Cy3-labeled) and Rb/K (Cy5-labeled) RNA. Triplicate arrays were performed with three independently isolated pools of K and Rb/K RNA, and t-tests were used to test for significance of differences.

Statistical Analysis

When applicable, a one-way ANOVA was used to test for significance of differences using Sigma Plot 11.0 Software (Systat Software, Inc, Chicago, IL). Kaplan-Meyer survival analyses were also done using Sigma Plot.

References

1. Carrière C, Seeley ES, Goetze T, Longnecker DS, Korc M. The Nestin progenitor lineage is the compartment of origin for pancreatic intraepithelial neoplasia. Proc Natl Acad Sci 2007;104:4437-4442.
2. Kleeff J, Korc M. Up-regulation of Transforming Growth Factor (TGF)-β Receptors by TGF-β1 in COLO-357 Cells. J Biol Chem 1998;273:7495-7500.
3. Seeley ES, Carrière C, Goetze T, Longnecker DS, Korc M. Pancreatic Cancer and Precursor Pancreatic Intraepithelial Neoplasia Lesions Are Devoid of Primary Cilia. Cancer Res. 2009;69:422-430.
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6. Ji B, Tsou L, Wang H, Gaiser S, Chang DZ, Daniluk J, Bi Y, Longnecker DS, Logsdon CD. Ras activity levels control the development of pancreatic diseases. Gastroenterology 2009;137:1072-1082.
7. Livak KJ, Schmittgen TD. Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the $2^{-\Delta\Delta CT}$ Method. Methods 2001;25:402-408.
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Supplemental figures and legends

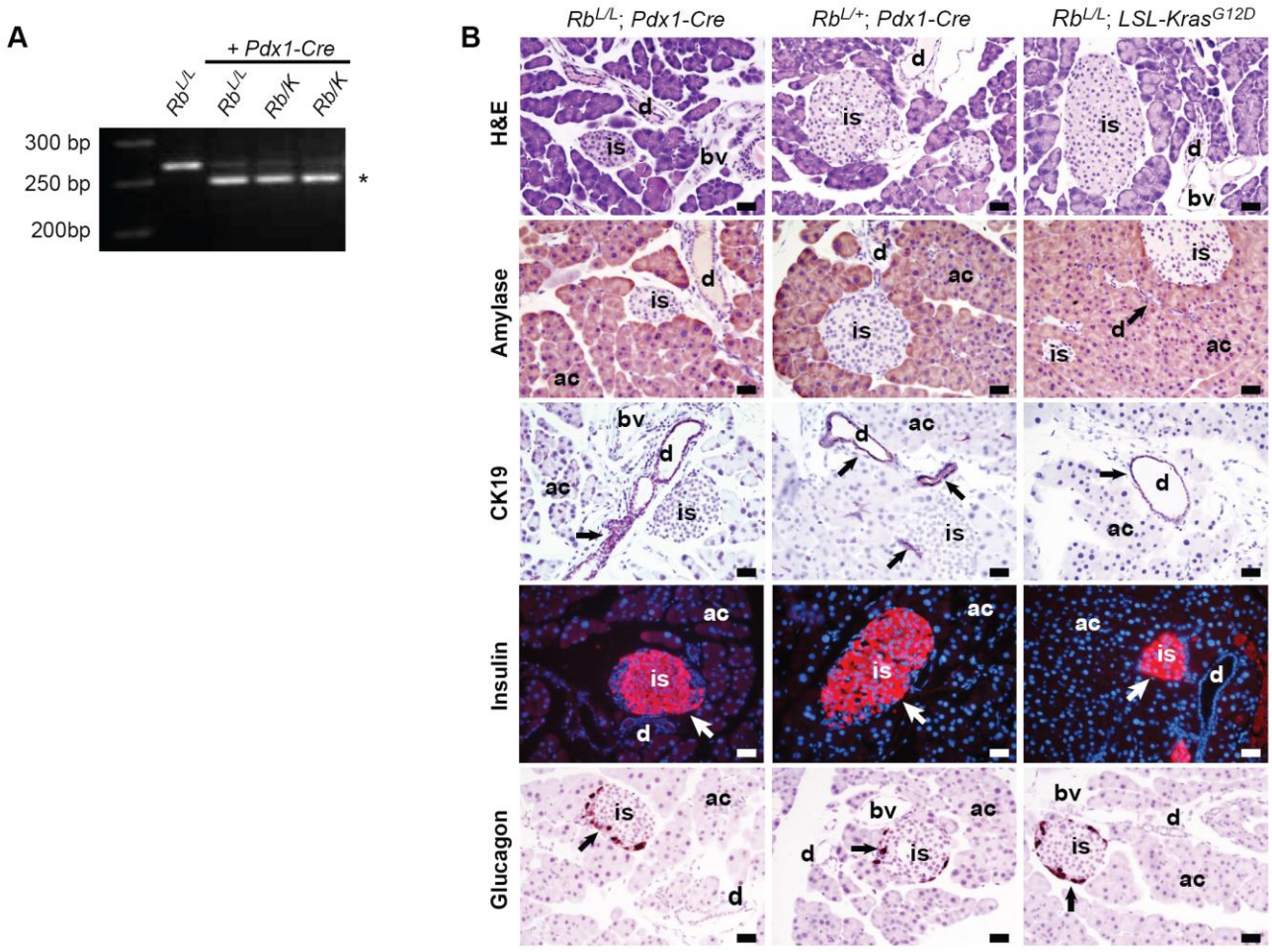


Figure S1: *Rb* deletion does not alter pancreatic cytoarchitecture.

(A) PCR using genomic DNA isolated from pancreata showed that *Pdx1* promoter-driven Cre recombined the floxed *Rb* alleles with ~80% efficiency (* indicates recombined *Rb*, higher band is non-recombined). In the absence of *Pdx1-Cre*, *Rb* recombination did not occur. (B) No differences were observed between *Pdx1-Cre;*$Rb^{L/L}$, *Pdx1-Cre;*$Rb^{L/+}$ and $Rb^{+/+}$ mice by H&E, or immunostaining for insulin, glucagon and amylase. (d: ducts; is: islets; bv: blood vessels; ac: acini).

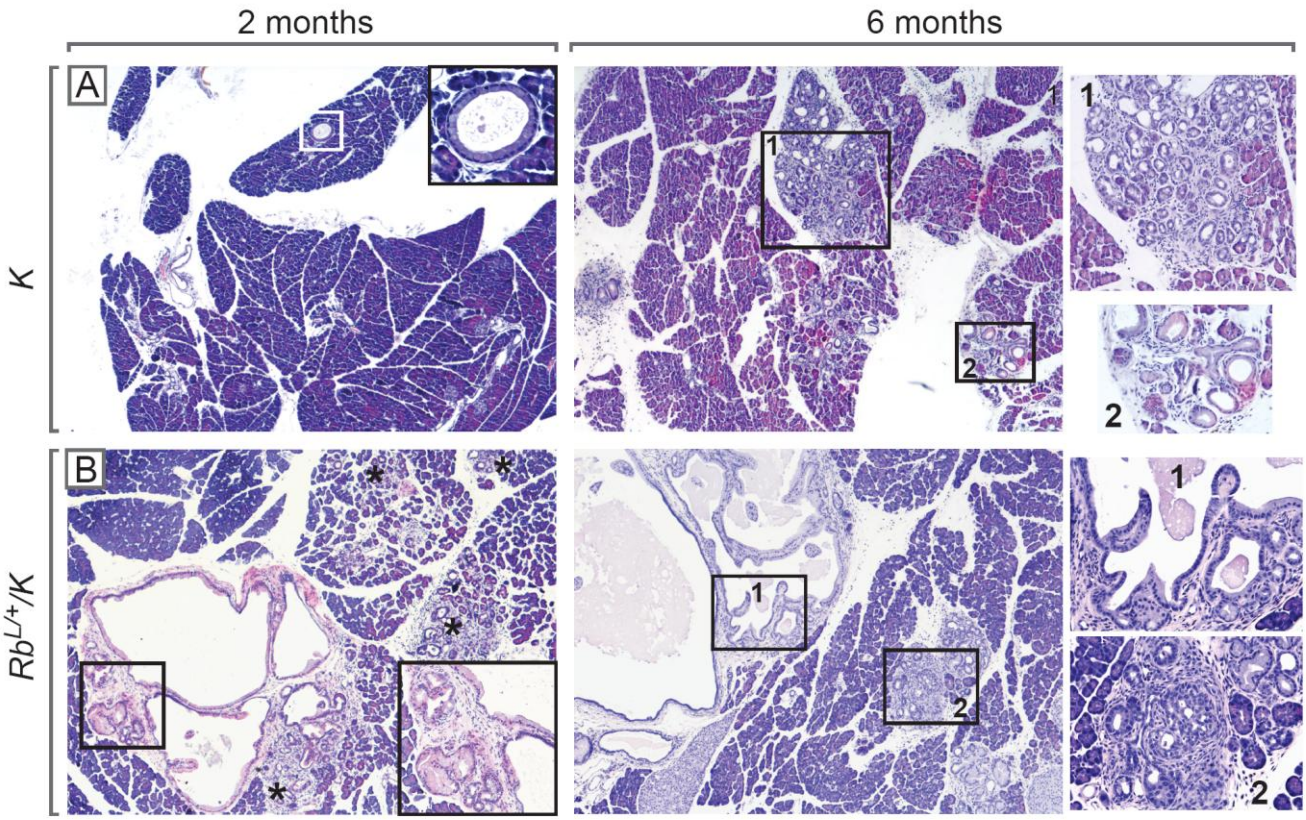


Figure S2: Accelerated development of pancreatic neoplasms in $Rb^{L/+}/K$ mice.

At 2 months postnatal (left panels), *K* pancreata (A) present rare, isolated low-grade PanIN, while $Rb^{L/+}/K$ pancreata (B) have advanced lesions (inset), cystic neoplasms and ADM foci (*). By 6 months (center and right panels), *K* pancreata (A) only have ADM foci (1) in addition to low-grade PanIN (2), while $Rb^{L/+}/K$ pancreata (B) have high numbers of ADM foci (2), and large cysts with advanced dysplasia (1).

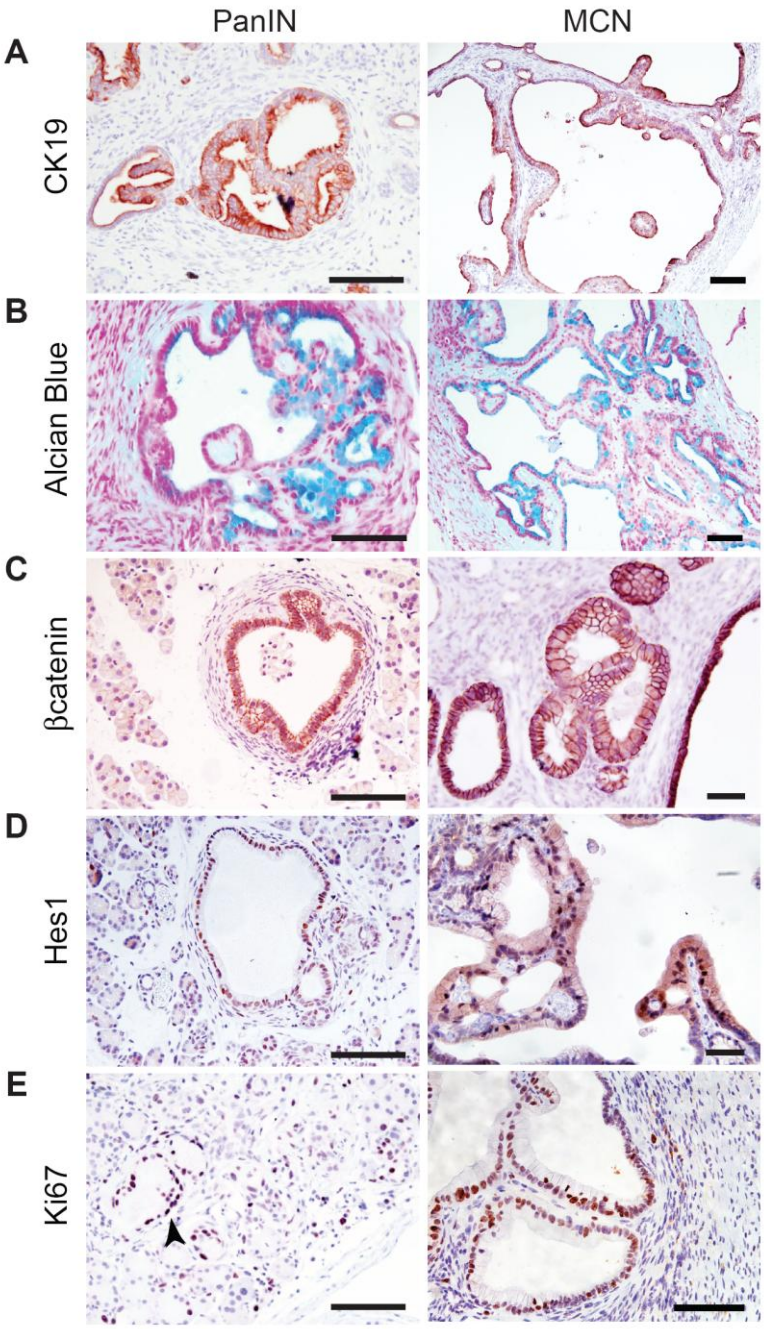


Figure S3: Histological features of PanIN and MCN-like lesions in *Rb/K* pancreata.

Both lesion types are of epithelial origin (A; CK19-positive), accumulate mucins (B; Alcian Blue-positive staining), express high levels of β–catenin (C) and Hes1 (D), and are highly proliferative (E; Ki67-positive, arrowhead).

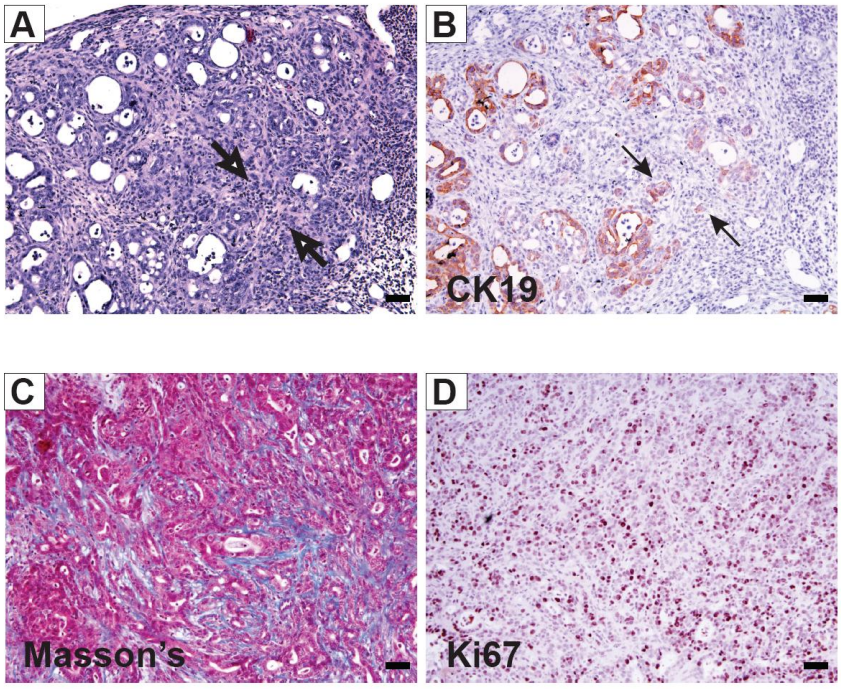


Figure S4: Characteristics of high-grade lesions and PDAC in *Rb/K* pancreata.

(A-B) High-grade lesions display microinvasion (A, arrows) as shown by presence of CK19-expressing cells (B, arrows) within the adjacent stroma. (C-D) Masson's trichrome staining (C) shows that PDAC accumulates collagen, and is highly proliferative as shown by abundance of Ki67-expressing cells (D).

Table S1: *Rb/K* mice develop acute pancreatic inflammation in early stages of cancer.

Genotype	diffuse acute inflammation	focal inflammation associated with ADM/lesions/PDAC
Pdx1-Cre; LSL-$Kras^{G12D}$;$Rb^{L/L}$		
2-3 weeks (n=5)	4/5	5/5
1 month (n=11)	6/11	10/11
2 months (n=10)	1/10	9/10
3 months (n=13)	0/13	13/13
4-5 months (n=6)	0/6	6/6
Pdx1-Cre; LSL-$Kras^{G12D}$;$Rb^{L/+}$		
2-3 months (n=7)	0/7	6/7
4-5 months (n=10)	0/10	10/10
6-8 months (n=5)	1/5	5/5
Pdx1-Cre; LSL-$Kras^{G12D}$		
2 months (n=8)	0/8	4/8
2-4 months (n=16)	0/16	12/16
4-6 months (n=19)	0/19	13/19
6-10 months (n=16)	0/16	15/16

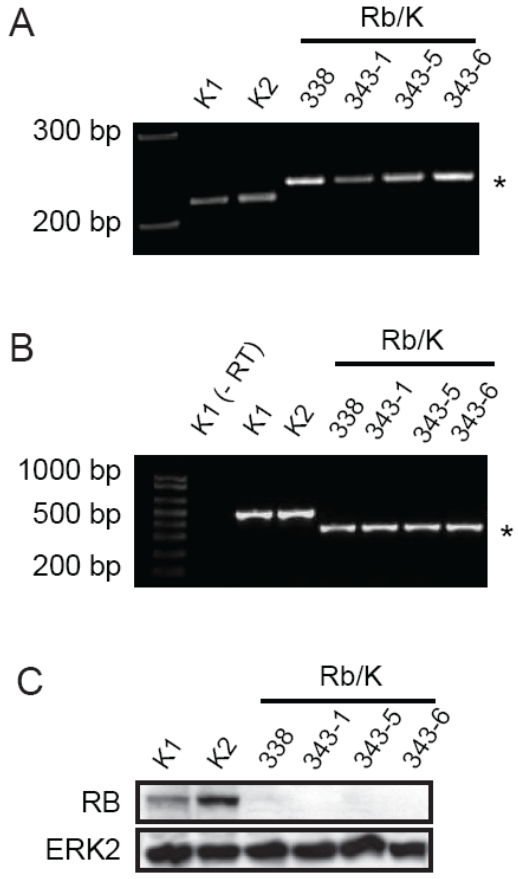


Figure S5: *Rb* recombination and RB expression in pancreatic cell lines.

(A) PCR on genomic DNA shows that *Rb* was recombined in all Rb/K cell lines (*). Only wild-type *Rb* was detected in K1 and K2 cell lines (lower band). (B) RT-PCR shows that exon 19 was deleted from *Rb* in all Rb/K cell lines (*), but not in K cells. K1 cells were used as a control to show that, in the absence of reverse transcriptase (-RT), there is no PCR product. (C) Consistent with the recombination data, RB is not expressed in Rb/K primary cell lines.

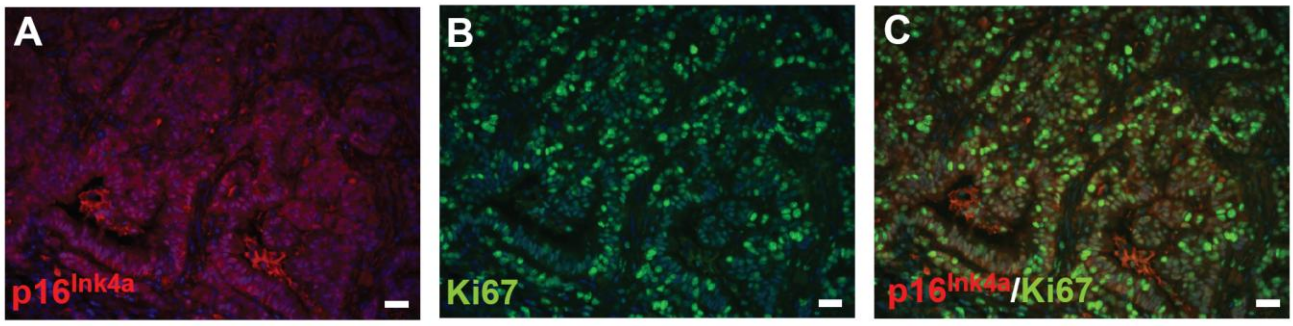


Figure S6: In *Rb/K* PDAC, $p16^{Ink4A}$ is absent.

(A-C) Co-immunofluorescence of $p16^{Ink4A}$ (A, red) and Ki67 (B, green) shows that in PDAC, most cancer cells express Ki67, but not $p16^{Ink4A}$ (C).

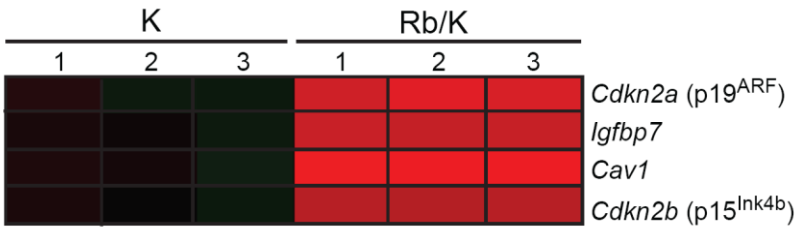


Figure S7: Senescence-associated genes are highly elevated in Rb/K cells.

A heatmap showing that in Rb/K cells (red), *Cdkn2a*, *Igfbp7*, *Cav1* and *Cdkn2b* are significantly elevated ($P<.001$). Shown are the results from triplicate arrays (1-3). When assayed by qRT-PCR, these genes were also significantly up-regulated in Rb/K cells (not shown).

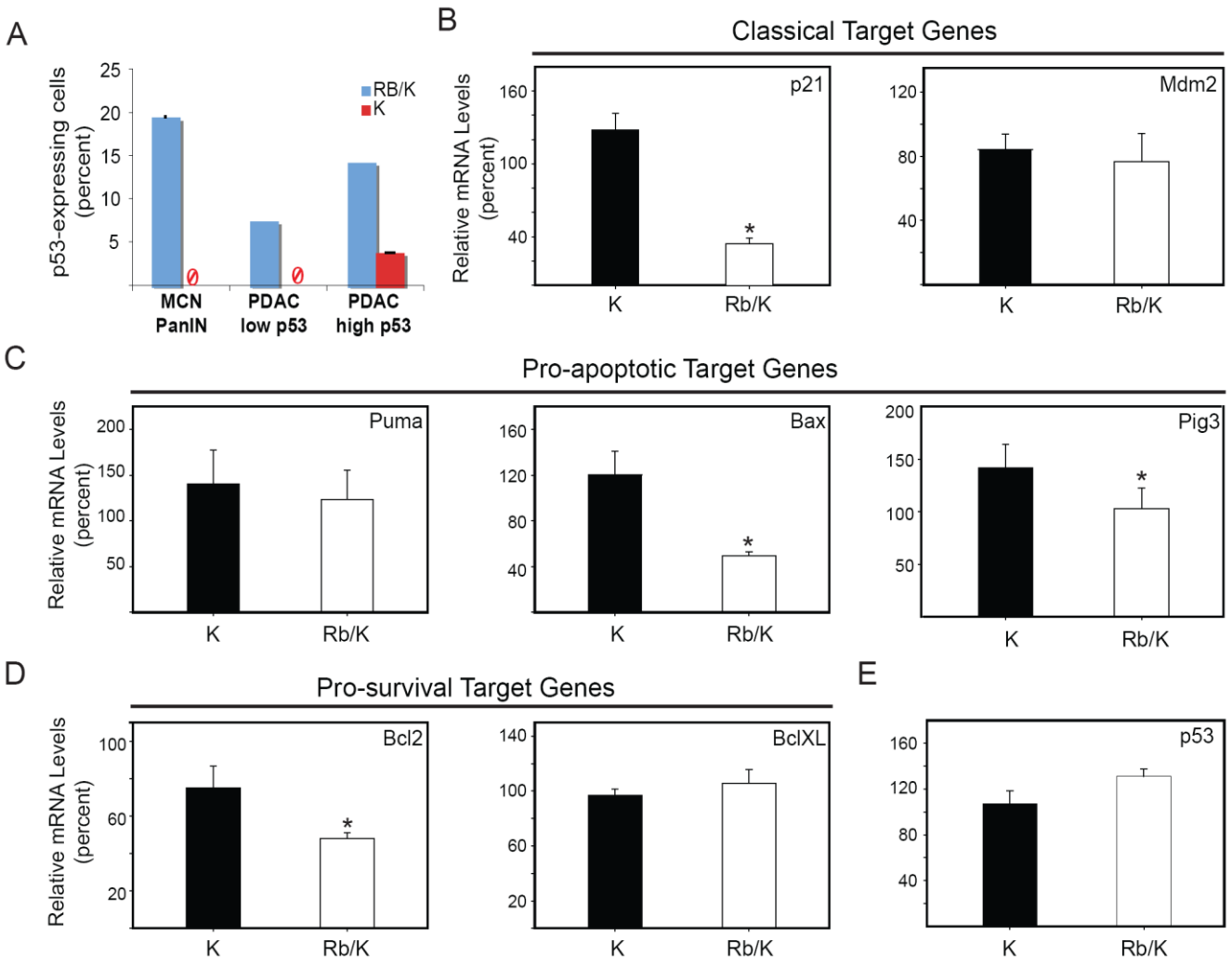
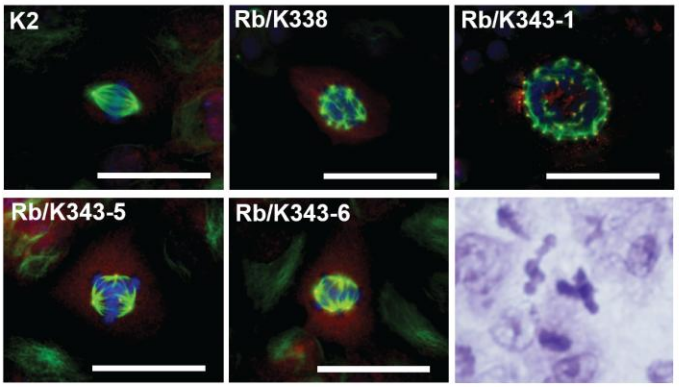


Figure S8: p53 stabilization does not lead to increased expression of different classes of target genes.

(A) Quantitation of p53 expressing cells shows that p53 is highly up-regulated in *Rb/K* PanIN, MCN-like lesions and PDAC. PDAC present areas of high and low p53 expression, but in both cases, p53 expression remains higher in *Rb/K* pancreata. Epithelial nuclei in at least 10 random fields for each genotype and lesion type were counted, and the ratio of p53 positive nuclei versus total nuclei was calculated. An ANOVA was used to test for significant differences. (B-D) qRT-PCR of an array of p53 target genes. Classical targets (*p21* and *Mdm2*), pro-apoptotic targets (*Bax, Puma and Pig3*) and pro-survival targets (*Bcl2* and *Bcl-XL*), are not up-regulated in Rb/K cells. Some genes (*p21*, *Bax* and *Bcl2*) are significantly down-regulated in Rb/K cells. (* $P < .05$) (E) p53 mRNA levels are similar in K and Rb/K cells.

A



cell line	K2	Rb/K338	Rb/K343-1	Rb/K343-5	Rb/K343-6
centrosome per mitosis +/- SEM	2	3.03 +/- 0.32	3.03 +/- 0.44	2.5 +/- 0.15	2.36 +/- 0.21

B

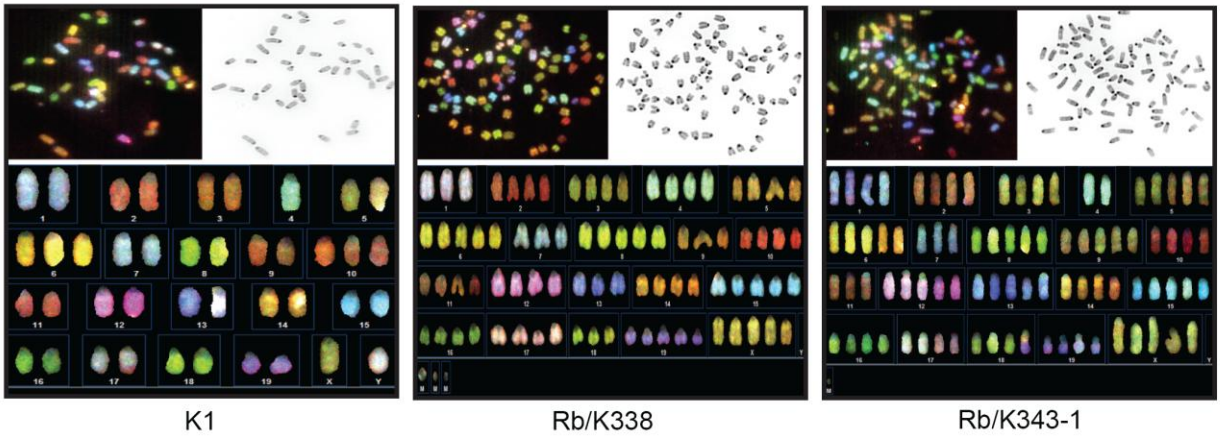


Figure S9: RB loss in the context of *Kras*^{G12D} activation leads to genomic instability.

(A) Co-immunofluorescence for γ-tubulin (red) and α-tubulin (green) shows that mitotic K cells have normal numbers of centrosomes (K2, top left panel), while Rb/K cells have multiple polar spindles and more than 2 centrosomes. H&E staining (bottom right panel) shows abnormal mitotic events in late stages in Rb/K pancreata. Scale bar, 25 μm. (B) SKY analyses on K and Rb/K cells, shows that Rb/K cells display marked chromosomal abnormalities, including marked chromosome amplification, and centric fragments. Shown are representative images from K1, and Rb/K338 and Rb/K343-1 cells. Rb/K343-6 cells display similar alterations (not shown).