

Supplemental Information

Loss of acinar cell IKK α triggers spontaneous pancreatitis in mice

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Supplemental Figures

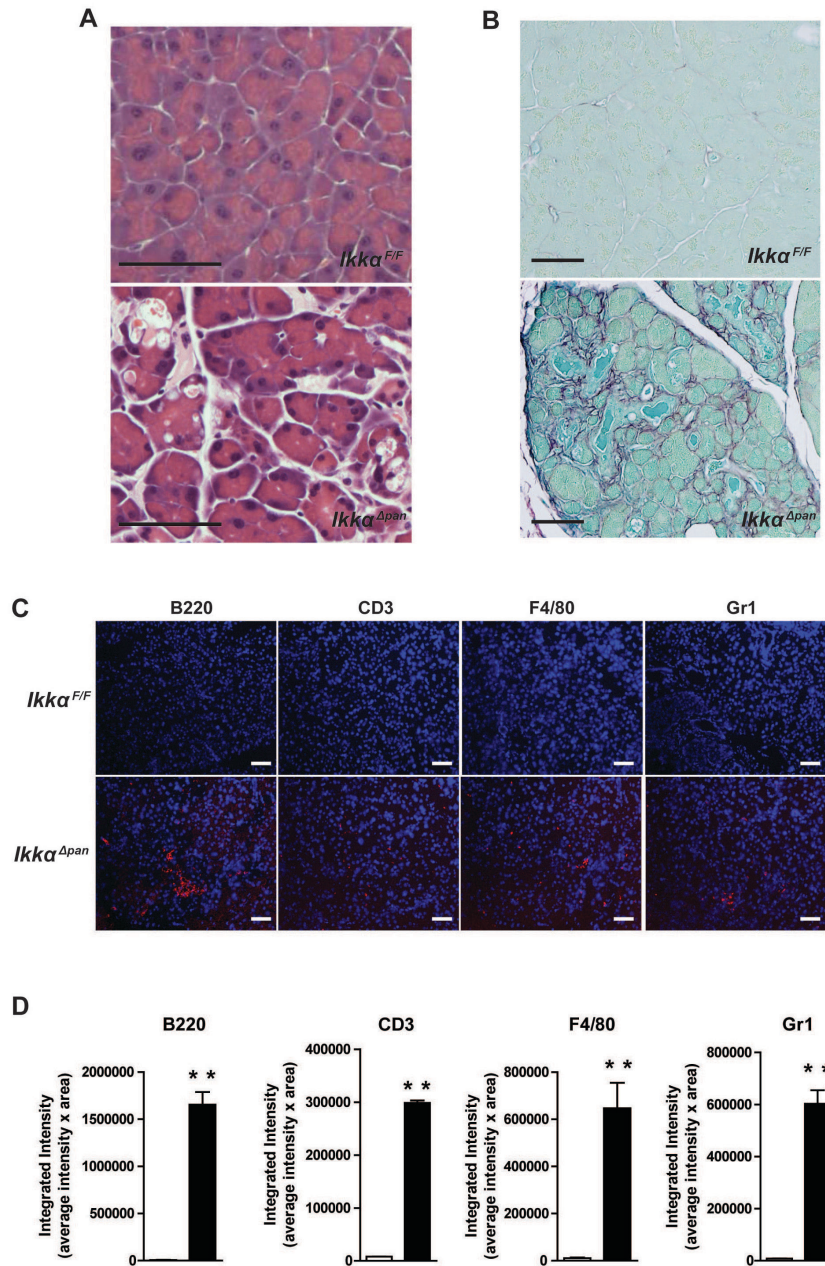


Figure S1

(A) High magnification image showing vacuolization in pancreatic acinar cells from 1 month old *Ikka^{Δpan}* mice. Scale bar: 50 μm. (B) High magnification images of Sirius Red stained pancreatic sections from 3 months old *Ikka^{Δpan}* mice. Scale bar: 50 μm. (C) Pancreas-specific *Ikka* ablation leads to immune cell infiltration. IF analysis of pancreatic sections from 5 months old *Ikka^{F/F}* and *Ikka^{Δpan}* mice with immune cell markers (B220- B cells, CD3 - T cells, F4/80 - macrophages, Gr1 – neutrophils). Scale bar: 50 μm. (D) Quantification of immune cell infiltration in pancreata of 5 months old *Ikka^{F/F}* and *Ikka^{Δpan}* mice. Results are means ± s.e.m. **, p<0.01.

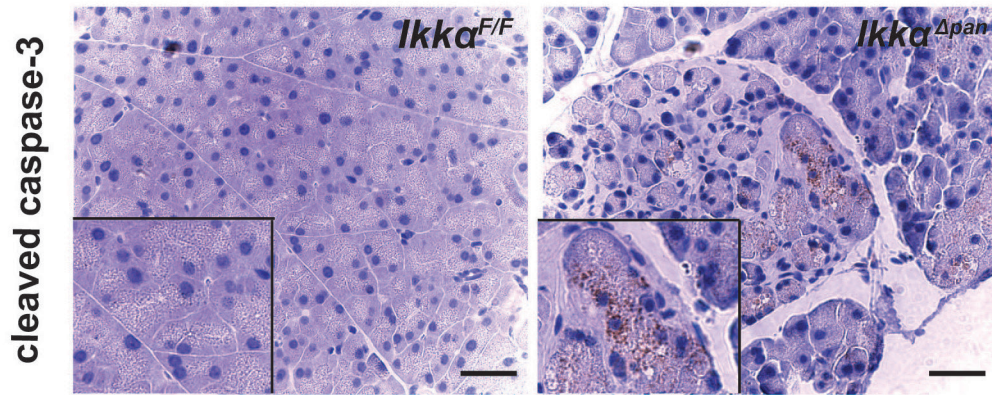


Figure S2

IHC staining for cleaved caspase-3 in pancreatic sections from 3 months old *Ikka^{F/F}* and *Ikka^{Δpan}* mice. Scale bar: 50 μm.

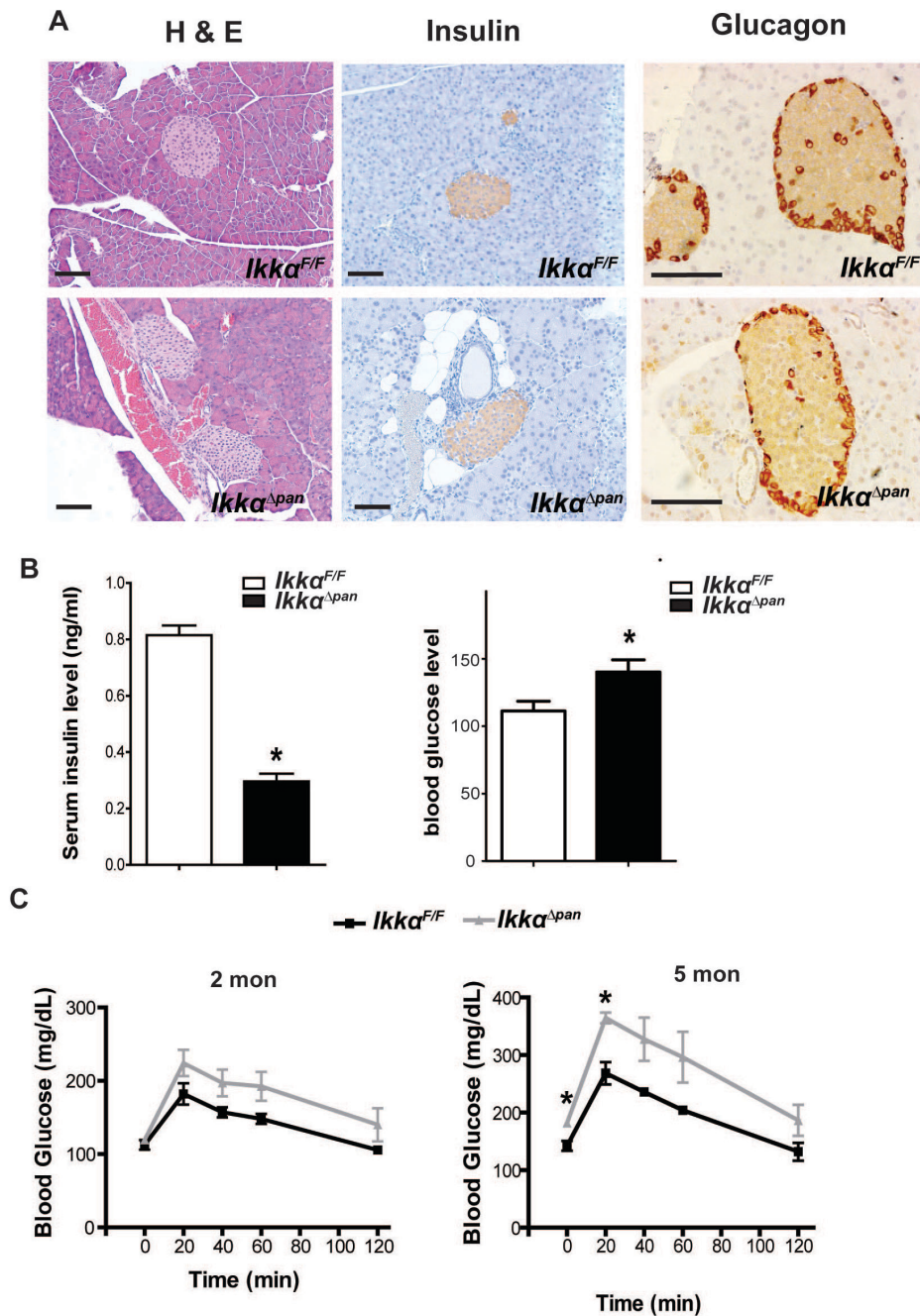


Figure S3

Endocrine deficiency in *Ikka^{Δpan}* mice. **(A)** H&E and IHC stainings of pancreatic sections from 3 months old *Ikka^{F/F}* and *Ikka^{Δpan}* mice. Scale bar: 50 μm. **(B)** Serum insulin and blood glucose in 3 months old *Ikka^{F/F}* and *Ikka^{Δpan}* mice fasted for 8 hrs before measurement. **(C)** Glucose tolerance test (GTT) was performed on *Ikka^{F/F}* and *Ikka^{Δpan}* mice of the indicated ages. Results are means ± s.e.m. $n=4-6$ mice per condition. * $p<0.05$.

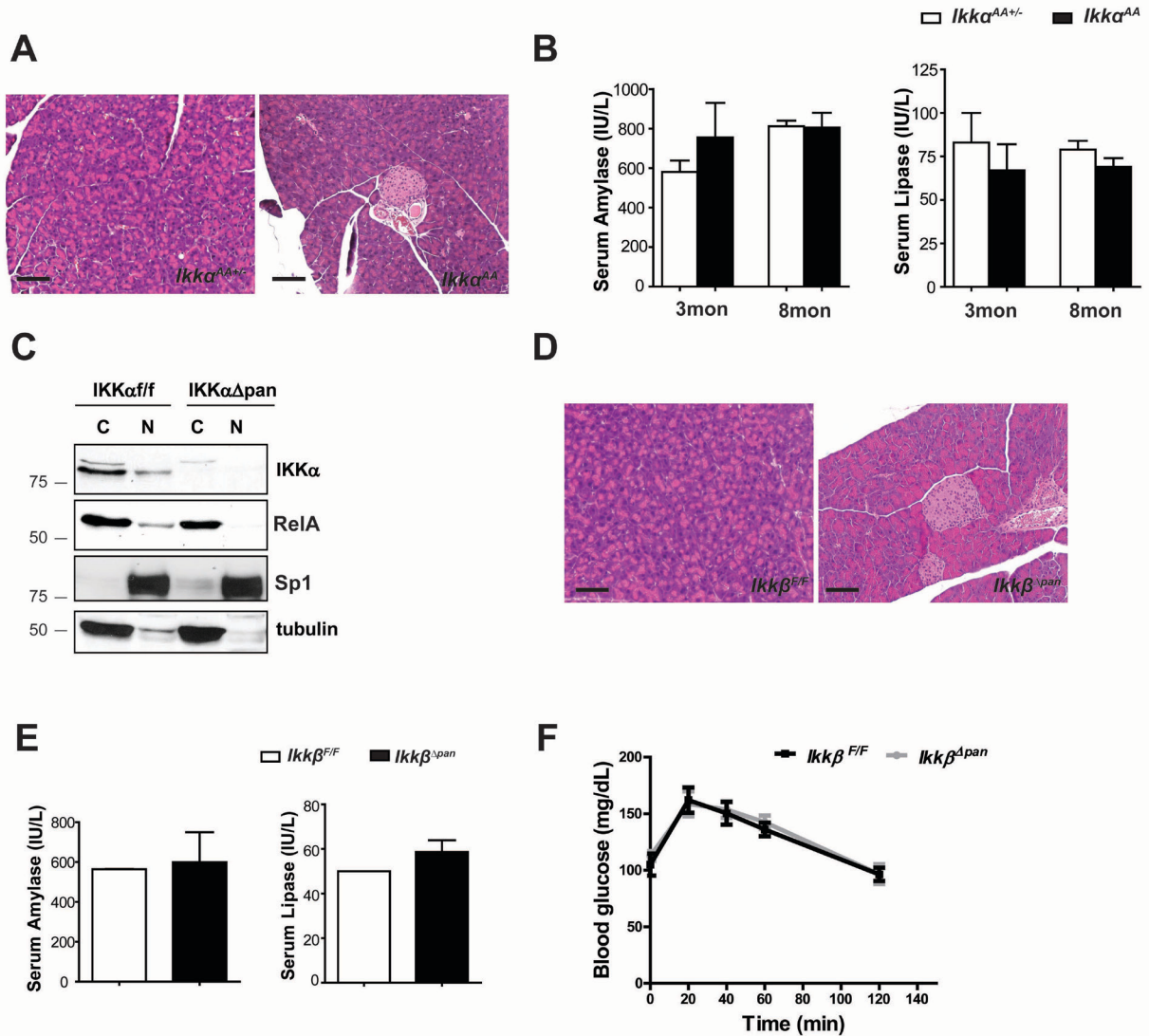


Figure S4

Ikkα^{AA} or *Ikkβ*^{Δpan} mice do not show any apparent pancreatic abnormalities. (A) Pancreatic histology of 3 months old *Ikkα*^{+/AA} and *Ikkα*^{AA/AA} mice (H&E staining). Magnification: 200x. (B) Serum amylase and lipase concentrations in 3 and 8 months old *Ikkα*^{+/AA} and *Ikkα*^{AA/AA} mice. (C) Analysis of RelA nuclear abundance in pancreata of 2 months old *Ikkα*^{F/F} and *Ikkα*^{Δpan} mice (C-cytosolic, N-nuclear fraction). Molecular weight markers in KDa are indicated. Proteins were separated by SDS-PAGE and immunoblotted with the indicated antibodies. (D) Pancreatic histology of 3 months old *Ikkβ*^{F/F} and *Ikkβ*^{Δpan} mice (H&E staining). Magnification: 200x. (E) Serum amylase and lipase concentrations in 5 months old *Ikkβ*^{F/F} and *Ikkβ*^{Δpan} mice. (F) GTT tests of 5 months old *Ikkβ*^{F/F} and *Ikkβ*^{Δpan} mice. Results are means ± s.e.m. *n*=5 mice per condition. Scale bar: 50 μm.

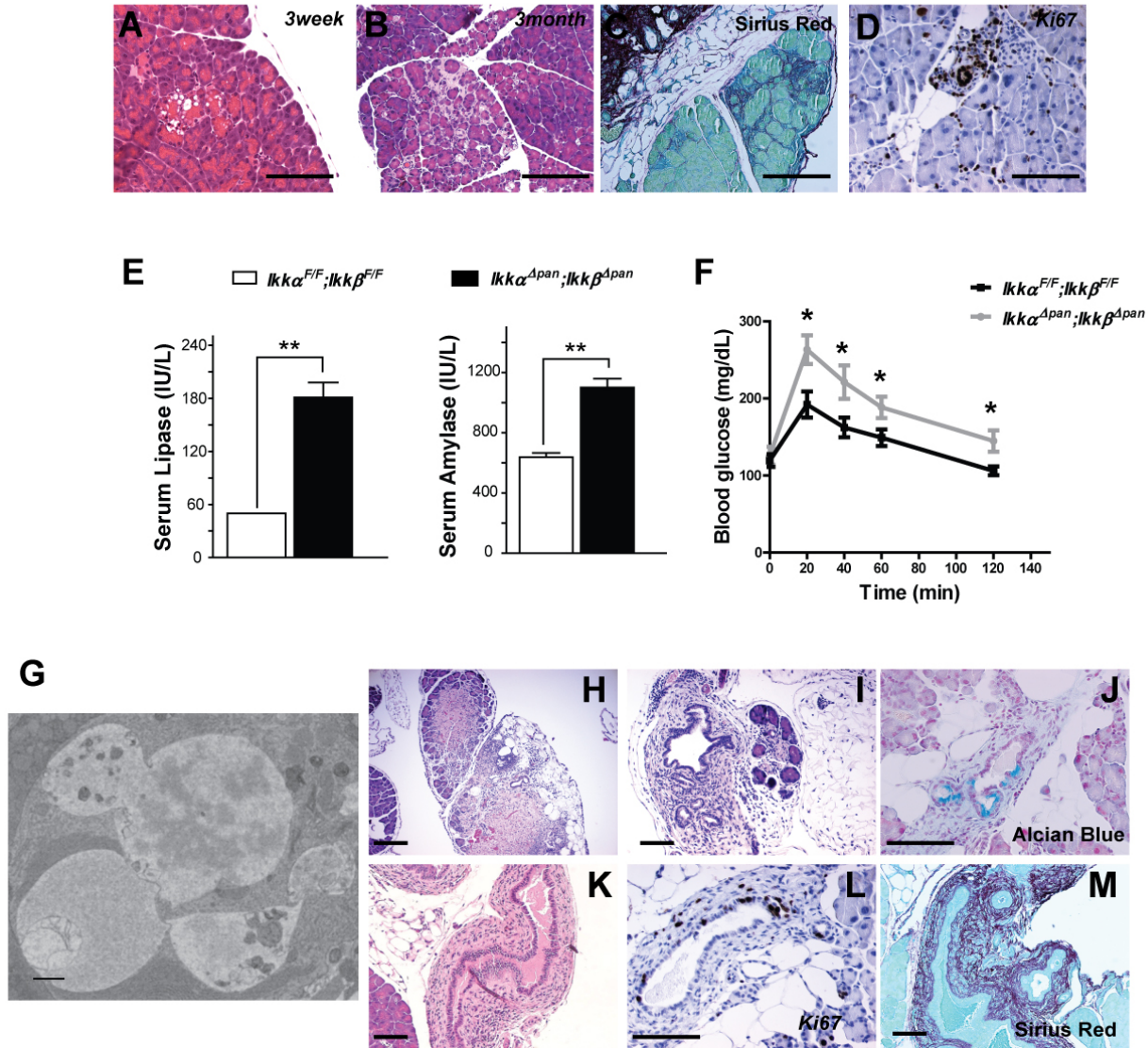


Figure S5

Additional IKK β deletion does not ameliorate pancreatitis in *Ikk α ^{Δ pan}* mice. (A-D) Pancreatic sections of 3 weeks or 3 months old *Ikk α ^{Δ pan};Ikk β ^{Δ pan}* mice stained with H&E (A,B), Sirius red (C) or Ki67 antibody (D). Note acinar cell vacuolization (A), fibrosis (B, C) and compensatory proliferation (D). Magnification: 400x. (E) Serum amylase and lipase concentrations in 6 weeks old *Ikk α ^{F/F};Ikk β ^{F/F}* and *Ikk α ^{Δ pan};Ikk β ^{Δ pan}* mice. (F) GTT tests of 3 months old *Ikk α ^{F/F};Ikk β ^{F/F}* and *Ikk α ^{Δ pan};Ikk β ^{Δ pan}* mice. (G) EM image of pancreatic acinar cells in 3 months old *Ikk α ^{Δ pan};Ikk β ^{Δ pan}* mice. Scale bar: 1 μ m. (H-M) Pancreatic sections from aged *Ikk α ^{F/F};Ikk β ^{F/F}* and *Ikk α ^{Δ pan};Ikk β ^{Δ pan}* mice stained with H&E (H,I,K), Alcian blue (J), Sirius red (M) or Ki67 antibody (L). Note acinar necrosis (H), fat replacement and PanIN lesions (I), mucin deposits in ducts (J), ductal hyperplasia (K), compensatory proliferation in ducts (L) and prominent periductal fibrosis (M) in *Ikk α ^{Δ pan};Ikk β ^{Δ pan}* pancreata. Magnification: 200x: (H), (I), (K) and (M); 400x: (J) and (L). Scale bars in A-D and H-M: 50 μ m. Results in (E) and (F) are means \pm s.e.m. *, $p < 0.05$; **, $p < 0.01$.

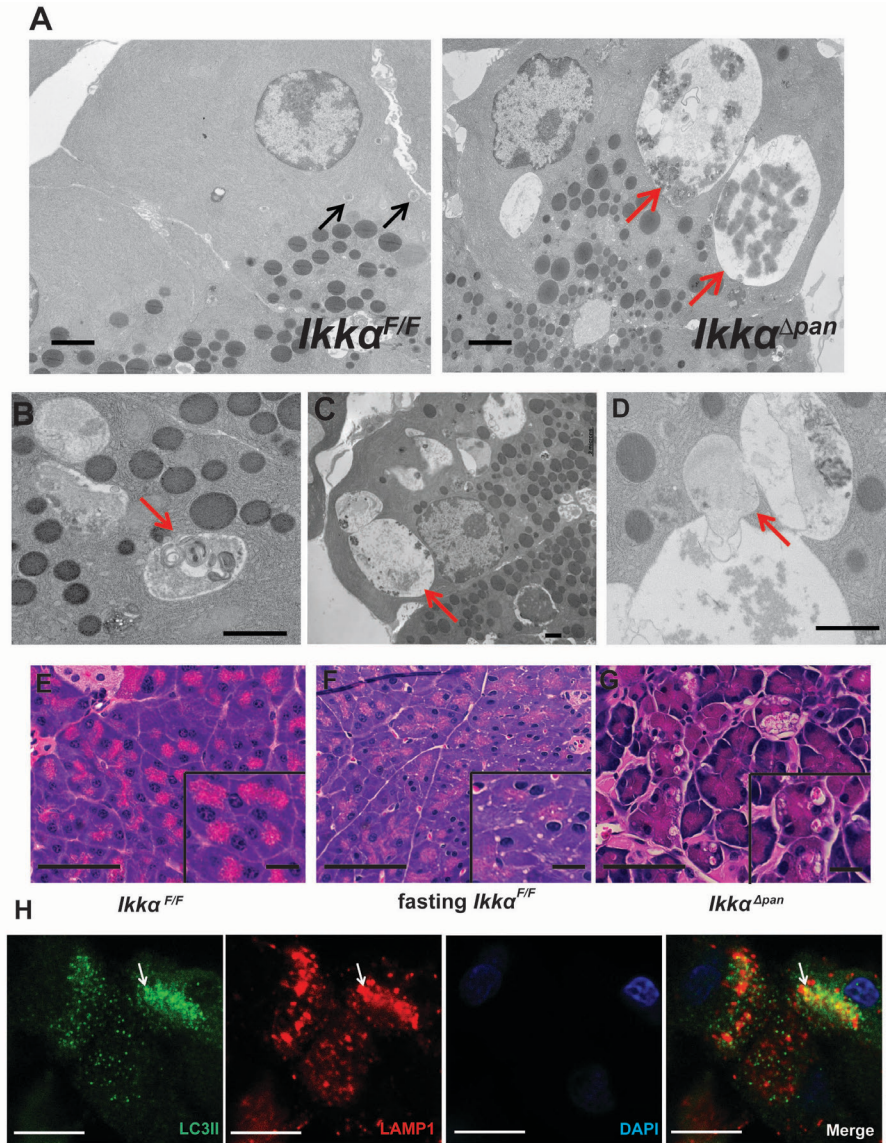


Figure S6

EM images of autophagic vacuoles and IF analysis of autophagosome-lysosome fusion in pancreatic acinar cells of *Ikka^{Δpan}* mice. **(A)** Enlarged EM images from Figure 3A showing accumulation of enlarged autolysosomes that contain partially degraded organelles, including ER and ribosomes, in *Ikka^{Δpan}* pancreatic acinar cells (red arrows), but not in *Ikka^{F/F}* mice (black arrows). Scale bars: 2 μ m. **(B-D)** EM images of *Ikka^{Δpan}* pancreatic acinar cells in 3 months old mice. Note the large autophagic vacuoles containing cargo at various stages of degradation (**B**: double-membraned autophagosomes, **C**: single-membraned autolysosomes and **D**: fusion of autophagic vacuoles). Scale bars, 1 μ m. **(E-G)** H&E staining of pancreatic sections from 3 months old non-fasting (**E**) or fasting (24 hrs) (**F**) *Ikka^{F/F}* mice and non-fasting *Ikka^{Δpan}* mice (**G**). Scale bar: 50 μ m. Inset scale bar: 10 μ m. **(H)** IF analysis with LC3 and LAMP1 antibodies of freshly isolated pancreatic acinar cells from 2 months old *Ikka^{Δpan}* mice. Note the colocalization of LC3II and LAMP1 (arrows). Scale bar: 10 μ m.

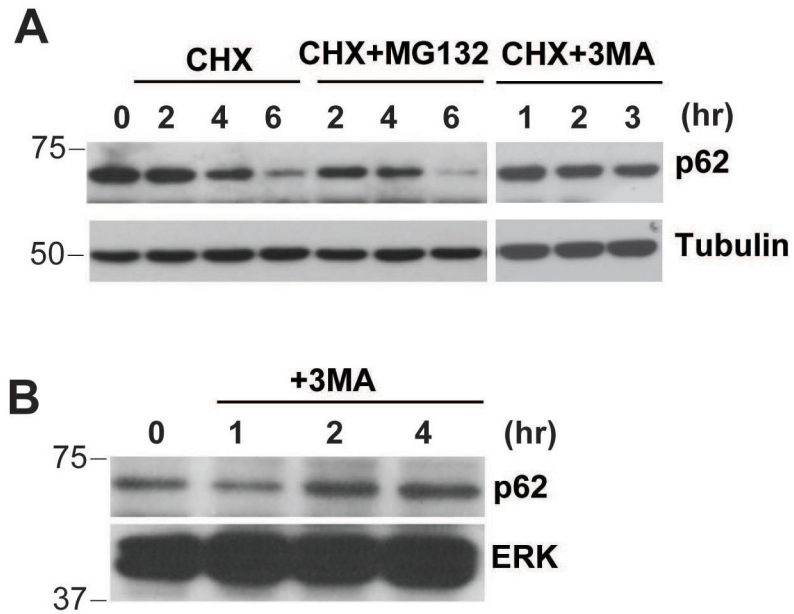


Figure S7

Autophagy is a major pathway for p62 degradation in acinar cells. **(A)** Freshly isolated pancreatic acinar cells from 2 months old *Ikk α ^{FF}* mice were incubated with cycloheximide (10 μ g/ml) in the absence or presence of the proteasome inhibitor MG132 (100 nM) or the autophagy inhibitor 3-methyladenine (3-MA; 10 mM). Protein lysates were prepared at the indicated times and subjected to IB analysis of the indicated proteins. **(B)** Freshly isolated pancreatic acinar cells from 2 months old *Ikk α ^{FF}* mice were incubated with 3-MA (10 mM). Protein lysates were prepared and analyzed as above.

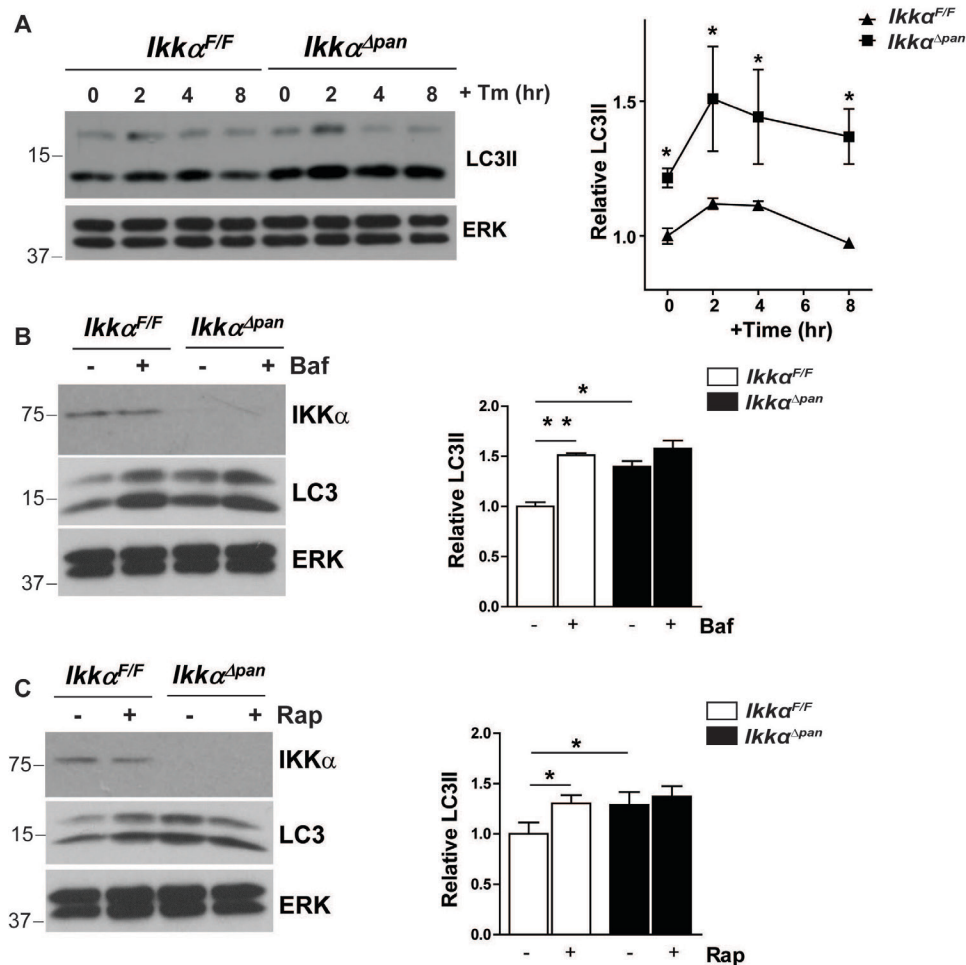


Figure S8

(A) Freshly isolated pancreatic acinar cells from 3 months old *Ikkα^{F/F}* and *Ikkα^{Δpan}* mice were incubated with 5 μM tunicamycin (Tm). Protein lysates were prepared at the indicated times, followed by IB analysis of LC3II and LC3I. Relative LC3II levels were quantified (*, $p < 0.05$). (B, C) Freshly isolated pancreatic acinar cells from 3 months old *Ikkα^{F/F}* and *Ikkα^{Δpan}* mice were incubated with bafilomycin A1 (Baf; 100 nM) for 4 hrs (B), or rapamycin (Rap; 200 nM) for 4 hrs (C). Protein lysates were prepared and analyzed as above. LC3II levels were quantified (*, $p < 0.05$; **, $p < 0.01$).

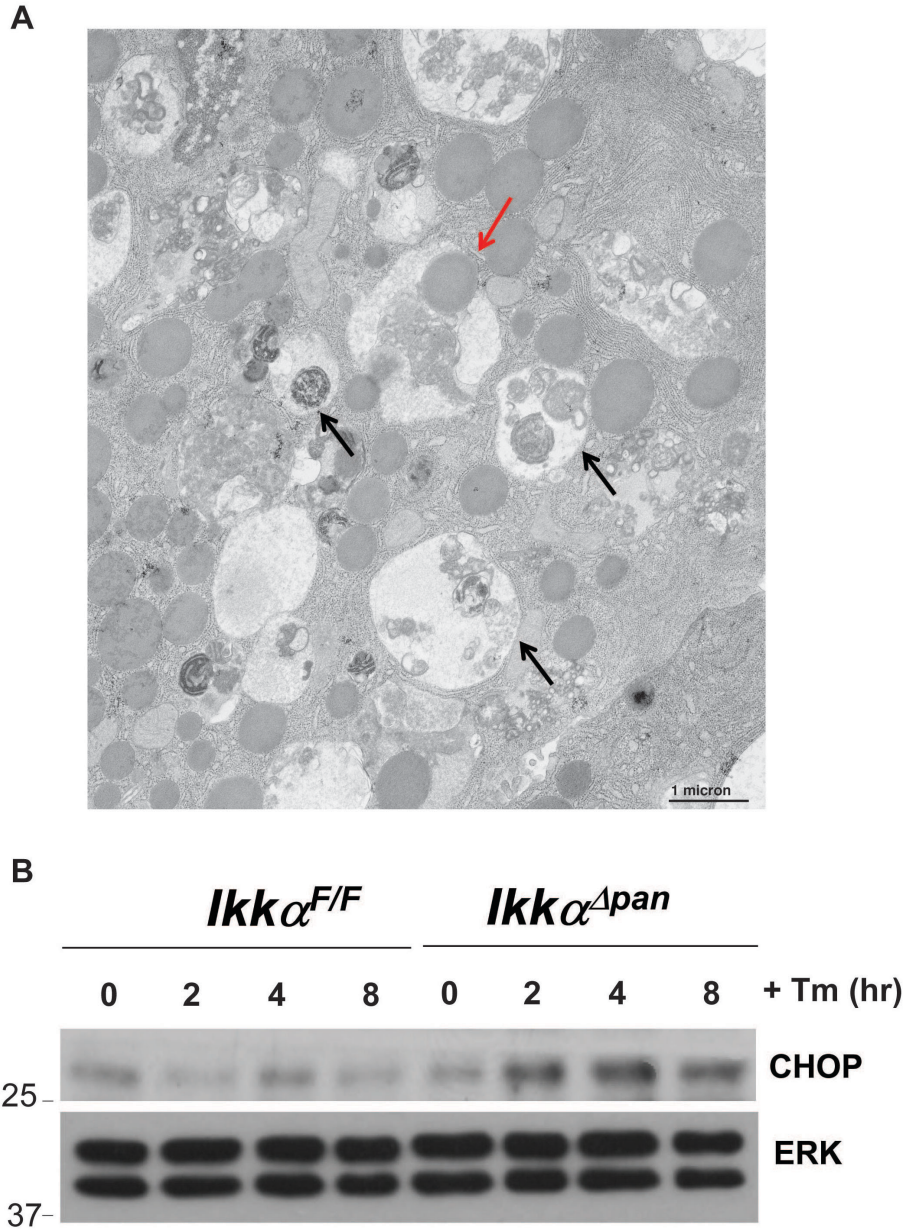


Figure S9

(A) Enlarged EM image from Figure 4C showing autophagic vacuoles in *Ikkα^{Δpan}* acinar cells that contain partially degraded ER and ribosomes (black arrows) or zymogen granules (red arrow). Scale bar: 1 μ m. **(B)** Ablation of IKK α facilitates CHOP induction in pancreatic acinar cells. IB analysis of CHOP expression following treatment of pancreatic acinar cells from 2 months old *Ikkα^{F/F}* and *Ikkα^{Δpan}* mice with 10 μ g/ml tunicamycin for the indicated times. Cell lysates were prepared and subjected to IB analysis. Note that since this is the same experiment as the one shown in Figure S8A, the loading control is identical.

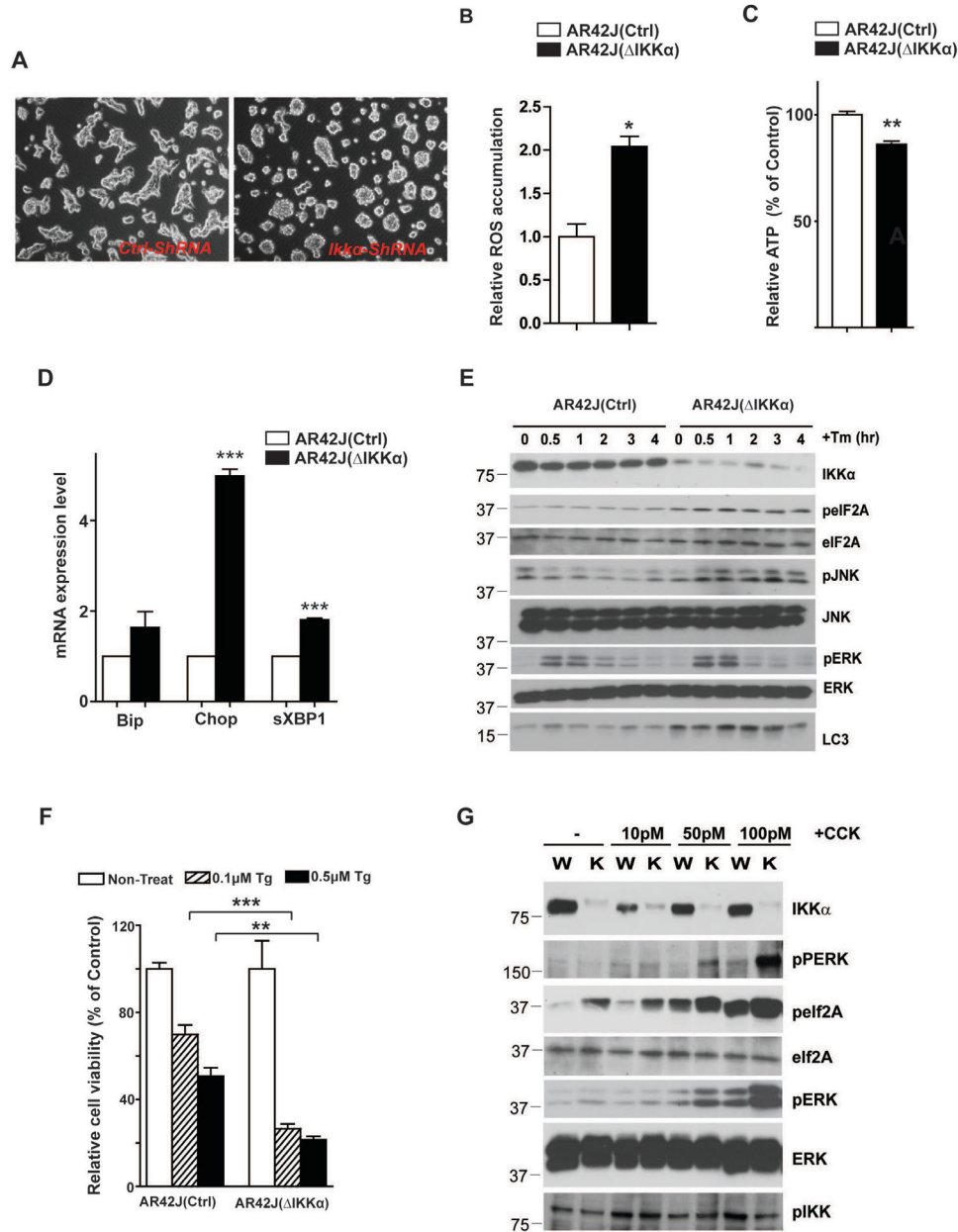


Figure S10

Loss of IKK α in pancreatic acinar cells results in oxidative and ER stress. **(A)** Morphology of rat pancreatic acinar AR42J cells stably transduced with lentiviruses containing control or *Ikk α* shRNAs. Magnification: 200x. **(B)** Analysis of ROS accumulation in AR42J (Ctrl) and AR42J (Δ IKK α) cells. **(C)** Relative intracellular ATP concentrations in AR42J(Ctrl) and AR42J(Δ IKK α) cells. **(D)** qRT-PCR of ER stress markers in AR42J(Ctrl) and AR42J(Δ IKK α) cells. **(E)** IB analysis of ER stress response and autophagy markers following treatment of AR42J(Ctrl) and AR42J(Δ IKK α) cells with 5 μ g/ml tunicamycin (Tm) for the indicated times. **(F)** Viability of AR42J(Ctrl) and AR42J(Δ IKK α) cells incubated the indicated concentrations of thapsigargin (Tg) for 24 hrs. **(G)** IB analysis of ER stress markers in AR42J(Ctrl) (W) and AR42J(Δ IKK α) (K) cells incubated with the indicated concentrations of cholecystokinin (CCK-8). Results are means \pm s.e.m. (n=3) *, p<0.05; **, p<0.01; ***, p<0.001.

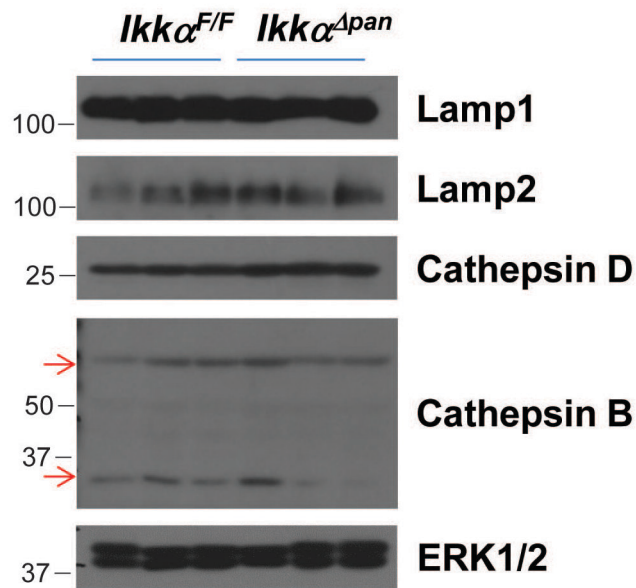
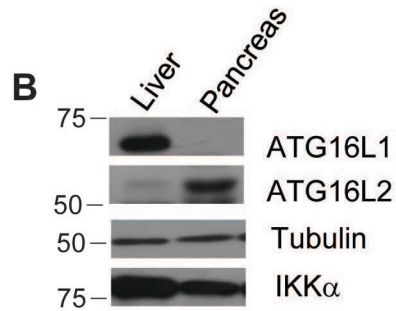
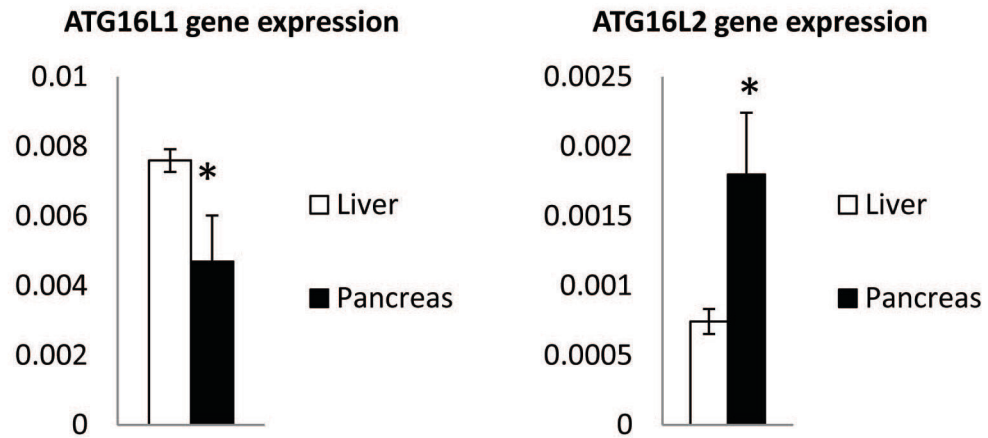


Figure S11

IB analysis of pancreatic lysates from 3 months old *Ikkα^{F/F}* and *Ikkα^{Δpan}* mice. Pancreatic lysates were subjected to IB analysis with the indicated antibodies. Each lane represents an individual mouse.

A**Figure S12**

Expression of ATG16L2 in pancreas and liver. RNA and protein were extracted from WT mouse pancreas and liver. **(A)** qRT-PCR analysis of *Atg16l1* and *Atg16l2* mRNA expression. Results are means \pm s.e.m. *, $p < 0.05$. **(B)** IB analysis of ATG16L1 and ATG16L2 protein expression.

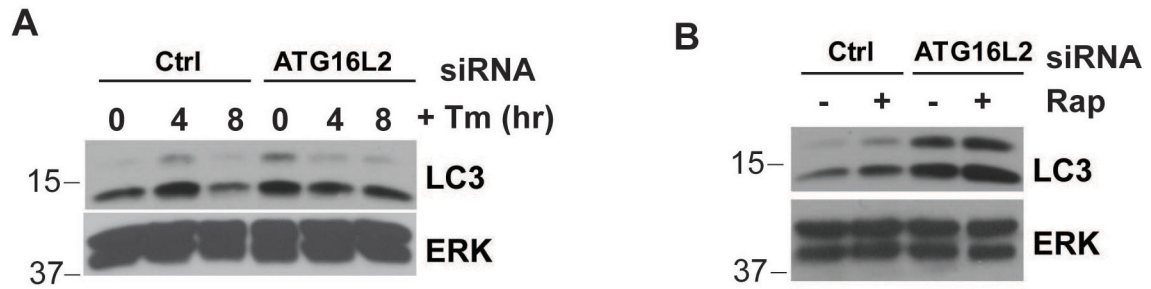


Figure S13

(**A, B**) Mouse pancreatic acinar cells were transfected with control siRNA or ATG16L2 siRNA, and treated with 10 µg/ml Tm (**A**) or Rap (200 nM), for 4 hrs (**B**). Protein lysates were prepared at the indicated times, followed by IB analysis of LC3II and LC3I. ERK was used as a loading control.

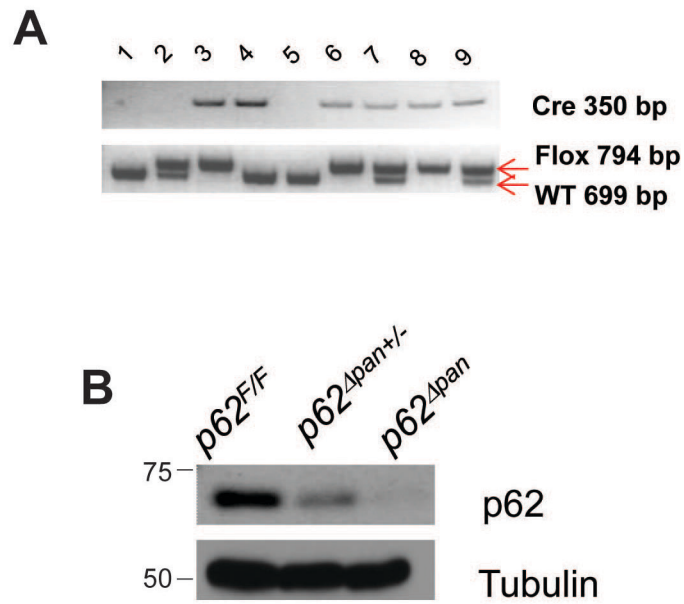


Figure S14

Generation of $p62^{\Delta pan}$ mice. **(A)** Genotyping of $p62^{\Delta pan}$ mice. **(B)** IB analysis of pancreatic lysates from $p62^{F/F}$, $p62^{\Delta pan+/-}$ and $p62^{\Delta pan}$ mice.

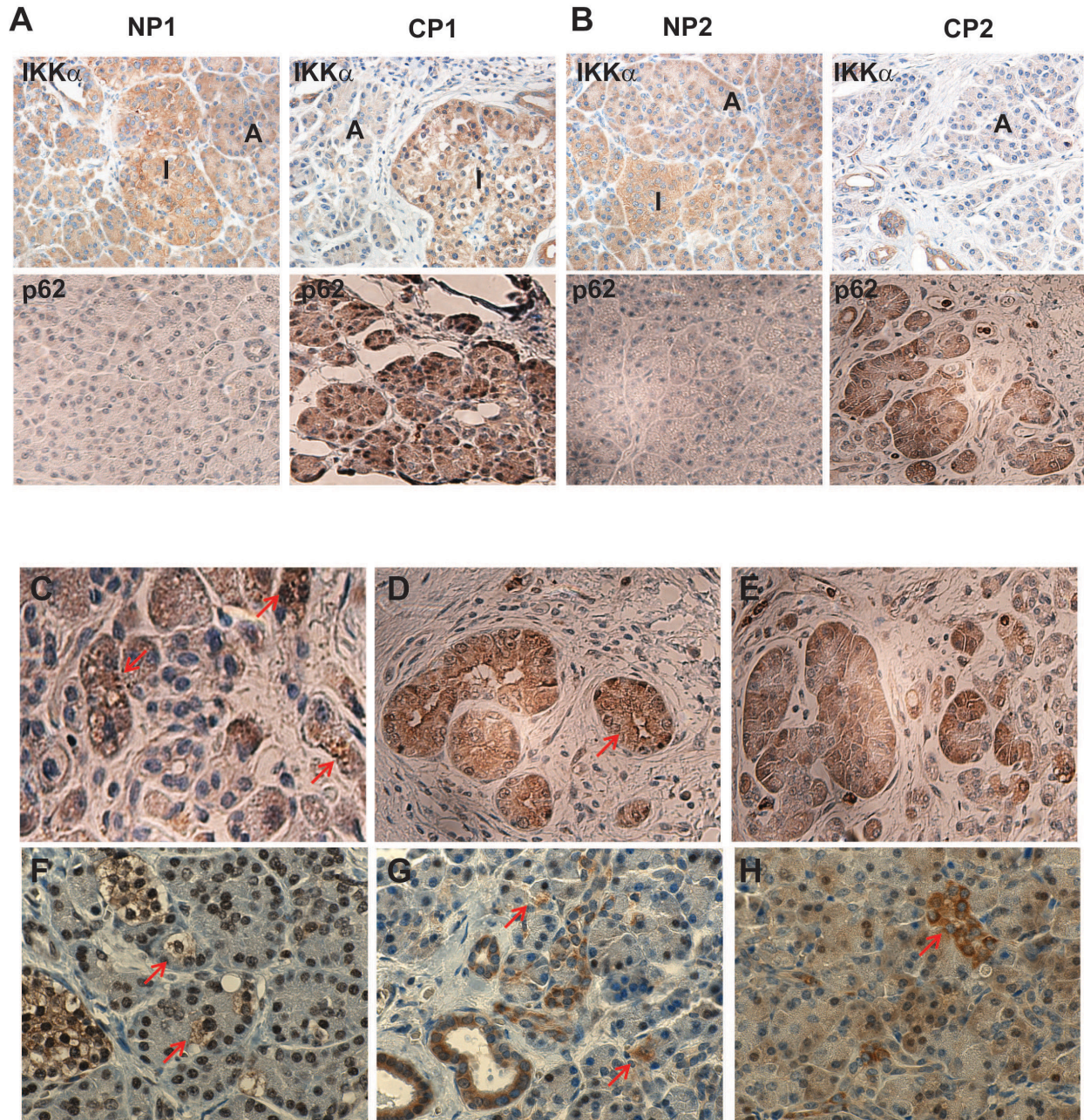


Figure S15

Downregulation of IKK α and upregulation of p62 in chronic pancreatitis. (A,B) IHC staining for IKK α and p62 of two pairs of paraffin-embedded human non-pancreatitis (NP) and chronic pancreatitis (CP) specimens. I, islet and A, acini. Note the downregulation of IKK α in acinar cells in CP, using the islets as internal controls. Magnification: 400x. (C-H) IHC staining of p62 in human pancreatic sections from 6 patients suffering from CP. The analysis includes only affected regions of the pancreas. Note p62 aggregates next to acinar cell vacuolization (red arrows). (C, F, G and H), Magnification: 630x. (D, E), Magnification: 400x.

Supplemental methods

Immunofluorescence. After xylene de-paraffinization and ethanol dehydration, tissue sections were incubated overnight with primary antibodies at 4°C, followed by incubation with secondary antibodies conjugated to Alexa-594 or Alexa-488. To prepare frozen sections, pancreas fragments were frozen in OCT compound (Sakura Finetek USA, Inc.) and sectioned with a cryomicrotome, air-dried and fixed with acetone. Frozen sections were processed for IF as above.

Electron Microscopy. Mice were fixed via cardiac perfusion at 37°C with 2% paraformaldehyde (freshly made), 2.5% glutaraldehyde in pH 7.4, 0.1 M sodium cacodylate buffer. Pancreata were washed three times with ice-cold buffer consisting of 0.1 M sodium cacodylate, 0.03% calcium chloride and postfixed with ice-cold 1% osmium tetroxide, 0.8% potassium ferrocyanide, 0.03% calcium chloride in 0.1 M sodium cacodylate for 1 hr on ice, washed three times with ice-cold distilled water, stained with 2% uranyl acetate at 4°C for 1 hr, dehydrated with graded ethanol solutions, and embedded in Durcupan ACM resin (Fluka, St. Louis, MO). Ultrathin (80 nm) sections were post-stained with uranyl acetate and lead salts prior to imaging with a JEOL 1200FX transmission EM operated at 80 kV.

Cell culture and lentiviral infection. Rat pancreatic AR42J acinar cells were maintained in F12/K medium supplemented with 20% FBS. shRNA to *Ikka* was generated as described (1), cloned into pLSLPw, and transfected into 293T cells. Lentivirus-containing supernatants were added to AR42J cells for 2 days with polybrene, and infected cells were selected in 5 µg/ml puromycin (Invitrogen).

GTT Assay. Mice were injected with glucose (2 g/kg, i.p.) after 6 hrs of fasting. Blood glucose was measured after 20, 40, 60 and 120 minutes using glucose meter (LifeScan).

Cell Fractionation. Cytosolic-nuclear fractionation was performed on freshly isolated pancreatic tissue using the Nuclear Extract Kit (Active Motif) according to the manufacturer's instructions.

ROS Assay. Intracellular ROS were measured using CM-H₂DCFDA (Invitrogen) and flow cytometry according to the manufacturer's instructions.

ATP Assay. Intracellular ATP was measured by the CellTiter-Glo assay using Luminescent CellTiter-Glo Cell Viability Assay Kit (Promega) according to the manufacturer's instructions.

Yeast Two-hybrid Screen. The Matchmaker Gal4 two-hybrid system 3 (Clontech) was used for screening according to manufacturer's instructions. The bait construct consisted of full-length human IKK α cloned between the EcoRI and BamHI sites of pGBKT7 with PCR-generated linkers. This bait was used to screen a mouse adult pancreas cDNA library cloned into the EcoRI site of pGAD10 (provided by Chandra L Tucker, Duke University).

Supplemental reference

1. Tan, W., Zhang, W., Strasner, A., Grivennikov, S., Cheng, J.Q., Hoffman, R.M., and Karin, M. 2011. Tumour-infiltrating regulatory T cells stimulate mammary cancer metastasis through RANKL-RANK signalling. *Nature* 470:548-553.