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Impaired JNK signaling cooperates with *Kras*^{G12D} expression to accelerate pancreatic ductal adenocarcinoma

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Abstract

The c-Jun N-terminal protein kinase (JNK) and its two direct activators, namely the mitogen-activated protein kinase (MAPK) kinase 4 (MKK4) and MKK7, constitute a signaling node frequently mutated in human pancreatic ductal adenocarcinoma (PDAC). Here we demonstrate the cooperative interaction of endogenous expression of Kras^{G12D} with loss-of-function mutations in *mkk4* or both, *mkk4* and *mkk7* genes in the pancreas. More specifically, impaired JNK signaling in a subpopulation of Pdx1-expressing cells dramatically accelerated the appearance of Kras^{G12D}-induced acinar-to-ductal metaplasia and pancreatic intraepithelial neoplasias, which rapidly progressed to invasive PDAC within 10 weeks of age. Furthermore, inactivation of *mkk4*/*mkk7* compromised acinar regeneration following acute inflammatory stress by locking damaged exocrine cells in a permanently de-differentiated state. Therefore, we propose that JNK signaling exerts its tumor suppressive function in the pancreas by antagonising the metaplastic conversion of acinar cells towards a ductal fate capable of responding to oncogenic stimulation.

Keywords

JNK; MKK4; MKK7; Kras; pancreatic cancer

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is one of the most lethal cancers with less than 4% of patients surviving past 5 years of diagnosis. More than 90% of human cases of

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invasive PDAC harbor activating mutations in the proto-oncogene *Kras* (1). According to the idea that *Kras* is a critical driver in disease initiation, endogenous expression of a $Kras^{G12D}$ mutant allele in the pancreas of genetically engineered mouse models faithfully reproduced the histological changes characteristic of human tumors (2). These included the formation of intraepithelial neoplasias (PanINs), the most common precursor lesions observed in human PDAC. Similar to human PanINs, PanIN lesions in mice (mPanINs) expressing physiological level of $Kras^{G12D}$ progress with age through defined histological and molecular stages (2). However, $Kras^{G12D}$ alone rarely induces full-blown pancreatic cancer (2, 3). Therefore, this model has also provided an excellent system to identify additional genetic alterations that interact with oncogenic Kras to accelerate the progression of mPanINs to invasive and metastatic PDAC.

Considering the ductal morphology of the carcinoma cells and their ability to express markers of ductal differentiation, the cell-of-origin susceptible to PanIN formation was initially believed to be within the ductal epithelium. However, this idea was challenged by the demonstration that induced expression of $Kras^{G12D}$ in differentiated ductal cells failed to yield a neoplastic phenotype (4). In contrast, spontaneous induction of mPanINs was observed following $Kras^{G12D}$ expression in the acinar compartment of young mice (5, 6). However, acinar cells in older animals become refractory to transformation by oncogenic *Kras* ($Kras^{G12D}$ or $Kras^{G12V}$), even in combination with inactivation of tumor suppressor genes such as *p53* and *Ink4A/Arf* (7-9). Interestingly, the resistance of adult mice to oncogenic *Kras* expression in acinar cells can be overcome by pancreatitis, one of the highest risk factor for PDAC development in human (7-10).

The importance of mutations known to occur in PDAC (*Kras, TP53, CDKN2A, SMAD4*) was reaffirmed by whole exome sequencing and copy number analyses of human pancreatic tumors (11, 12). In addition, these studies revealed novel significantly mutated genes, including a clustering of mutations in multiple components of the c-Jun NH_2-terminal kinase (JNK) signaling pathway. JNK was originally identified as the mitogen-activated protein kinase (MAPK) responsible for regulating by phosphorylation the activity of c-Jun (13, 14). This discovery was a major breakthrough considering that the stimulation of c-Jun activity was known to be essential for cooperating with Ha-*ras* in oncogenic transformation (15). Like other MAPKs, JNK is activated upon dual phosphorylation by MAPK kinases (MKKs), namely MKK4 and MKK7 (16). Forward genetic screens in mice carrying the $Kras^{G12D}$ allele and *Pdx1-Cre* transgene using Sleeping Beauty transposon mutagenesis confirmed the potential function of MKK4 as a tumor suppressor in pancreatic cancer (17, 18).

Although JNK is predicted to be an important component of signal transduction in *Ras*-mediated oncogenesis, the requirement of JNK signaling in pancreatic tumorigenesis has never been investigated *in vivo*. Consequently, we examined the effect of pancreas-specific *mkk4* and *mkk7* gene deletion in the context of endogenous $Kras^{G12D}$ expression and in the setting of pancreatitis to increase our molecular understanding by which activated Kras contributes to pancreatic cancer.

Materials and Methods

Mouse strains

The *Pdx1-Cre*^{ER} line was provided by Doug Melton (19), *Kras*^{G12D} knock in strain was provided by Tyler Jacks (20). Floxed (lox) *mkk4* and/or *mkk7* strains were generated in our laboratories (21, 22). *Pdx1-Cre*^{ER}*;mkk4/7*$^{+/lox}$ and *Kras*^{G12D}*;mkk4/7*$^{+/lox}$ mice were inter-crossed to generate control and experimental animals. Tamoxifen (14 μg; Sigma-Aldrich) was administrated by oral gavage to lactating dams 7 days post-parturition to induce Cre-mediated recombination of *lox*-alleles in offspring. The mouse strains were maintained in a pathogen-free facility at the University of Manchester Biological Safety Unit. All animal procedures were performed under license in accordance with the UK Home Office Animals (Scientific Procedures) Act (1986) and institutional guidelines.

Acute pancreatitis model

Five weeks old offspring received 6 hourly intra-peritoneal injection of PBS or caerulein (50 μg/kg body weight; Sigma-Aldrich) on 2 days separated by a 48 hours period.

Immunoblot analysis

Proteins were extracted by homogenizing tissue samples in RIPA buffer containing inhibitors of proteases and protein phosphatases. Extracts (20 μg) were resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and electrophoretically transferred to an Immobilon-P membrane (Millipore, Inc.). The membranes were saturated in 3% non-fat dry milk and probed overnight with antibodies (1:1000) to JNK (Santa Cruz Biotechnology), phospho (p)-JNK (Cell Signaling Technology), MKK4 (Cell Signalling Technology), MKK7 (Cell Signalling Technology) or tubulin (Sigma). Immunecomplexes were detected by enhanced chemiluminescence.

Protein kinase assay

Endogenous JNK activity was measured in pancreatic extracts (250 μg) following immunoprecipitation using the JNK1/3 antibody (Santa Cruz Biotechnology) in kinase buffer containing [γ-^{32}P] ATP (10 Ci/mmol) and 1 μg of GST-cJun. The radioactivity incorporated into the recombinant protein was quantified after SDS-PAGE by PhosphorImager analysis (21).

Immunohistochemical and immunofluorescence analyses

Freshly isolated biopsies of the pancreas were fixed in 10% Neutral-buffered Formulin (Sigma-Aldrich) for 16 hours and embedded in paraffin. 5 μm-thick sections were deparaffinized, rehydrated and treated in boiling sodium citrate buffer (10 mM pH 6.0) for 10 min to unmask antigens. Endogenous peroxidase activity was quenched by treating the slides with 3% hydrogen peroxide for 10 min. Sections were blocked in PBS containing 10% goat serum, 1% BSA and 0.1% Triton-X100 for 1 h at room temperature prior to being incubated overnight at 4°C with primary antibodies to amylase (1:50; Santa Cruz Biotechnology), β-catenin (1:1500; Cell Signaling Technology), CK19 (TromaIII 1:75; DSHB), MKK4 (1:50; Cell Signalling Technology), Notch ICD (1:200; Abcam), p-ERK1/2

(1:50; Cell Signaling Technology), p-JNK (1:50; Cell Signaling Technology), p-STAT3 (1:50; Cell Signaling Technology), Pdx1 (1:10000; Beta Cell Biology Consortium), smooth muscle actin (αSMA) (1:800; Abcam), Sox9 (1:2000; Millipore) or TGFβ (1:25; Abcam). After incubation with secondary biotinylated antibodies (1:200; Vector laboratories, UK), sections were processed using the Vectastain elite ABC kit (Vector laboratories, UK). The presence of the antigens was revealed using diaminobenzadine tetrachloride (DAB, brown; Vector laboratories, UK) and counter stained with nuclear red or with heamatoxylin (blue). Brightfield images were taken using a Zeiss Axio Scope A1 microscope. For immunofluorescence, amylase and CK19 were detected by Alexafluor goat anti-rabbit-488 (1:200, Invitrogen, UK) or donkey anti-rat-Cy3 (1:400, Jackson Immuno, USA), respectively. Images were captured using an Olympus BX51 microscope.

Evaluation of CK19 and amylase positive area

A minimum of 6 random and non-overlapping fluorescent images separated by a depth of 100 μm were collected from at least 3 mice per condition. ImageJ was used to calculate the percentage area covered by amylase or CK19 expression and the percentage area of co-localization via the “Colocalization Highlighter” ImageJ plugin. Measurements were normalized to pancreatic tissue area and expressed as a ratio.

Histopathological Evaluation

Scoring of PanIN lesions was conducted blind by a clinical pancreatic pathologist (R.F.T.M.). Three paraffin sections of at least 200 μm apart were visualized at x10 magnification and the most prominent histological phenotype of each view recorded (from ADM to PDAC). The 5 highest-grade lesions from each section were used to calculate the mPanIN score index as follows: 1 × (# PanIN-1A) + 2 × (# PanIN-1B) + 3 × (# PanIN-2) + 4 × (# PanIN3/PDAC). Scores can therefore range from 0 (no PanIN lesions) to 20 (five PanIN3/PDAC). The data are presented as an average across the three sections from at least two mice.

Statistical analysis

Statistical significance was determined using two-tailed student’s t test for two samples with unequal variance. For all mouse tissue experiments, images represent at least 3 mice.

Results

Generation of mouse strains with pancreas-specific *mkk4/mkk7* gene deletion

Conditional deletion of the *mkk4* and *mkk7* loci through Cre-mediated recombination generates bone-fides null alleles (23). Here, the promoter of the *Pdx1* gene was utilized to drive the expression of Cre in the pancreas of *mkk4/7*$^{lox/lox}$ mice. Pdx1 is expressed in acini and islets at all stages of embryonic development and in postnatal mice, while duct progenitors express Pdx1 only between E9.5 and E11.5 (19). To avoid potential developmental defects caused by early embryonic loss of MKK4 and MKK7, we used an inducible form of Cre, namely Cre^{ER}. Genetic recombination was induced in offspring through the milk of lactating dams that had received tamoxifen (TM) by oral gavage 7 days after parturition.

Immunoblot analysis with antibodies to MKK4 or MKK7 demonstrated that inactivation of the *mkk4* and *mkk7* genes only occurred in pancreata of homozygous *lox* littermates that had inherited the *Pdx1-Cre*^{ER} transgene (Fig. 1A). The residual level of MKK7 expression in pancreatic extracts derived from 2 out of 4 *Pdx1-Cre*^{ER}; *mkk4*$^{lox/lox}$;*mkk7*$^{lox/lox}$ animals tested confirms that Cre-mediated recombination is not 100% efficient. The selective loss of protein expression in the pancreas is shown by similar level of MKK4 in heart, spleen, liver and lung of both control and mutant mice (Fig. 1B). Furthermore, consistent with previous knowledge that MKK4 and MKK7 have both overlapping and non-redundant function (24), JNK activity decreased by around 50% in the absence of MKK4 or MKK7 expression and by around 80% when both genes were deleted (Fig. 1C).

Additionally, immunostaining of pancreatic sections with an antibody against MKK4 indicated that protein loss occurred in a mosaic fashion (Fig. 1D). According to the pattern of *Pdx1* gene expression (19), ducts remained positive for MKK4 (Fig. 1D). Overall, MKK4/7 deficiency in exocrine and endocrine tissues did not cause any gross pancreatic morphological defect, as demonstrated by histologically normal ducts, islets and acini in the pancreata of adult *Pdx1-Cre*^{ER};*mkk4*$^{lox/lox}$;*mkk7*$^{lox/lox}$ mice (Fig. 1E-G). This indicated that JNK signaling was not required for pancreatic homeostasis.

The loss of *mkk4* sensitizes the pancreas to Kras^{G12D}-induced PanIN formation

The *mkk4* locus has been identified as one of the most significantly mutated genes in human pancreatic cancer (11, 12). This led us to examine the functional consequence of *mkk4* loss-of-function mutation in mPanIN formation by crossing the *Pdx1-Cre*^{ER};*mkk4*$^{lox/lox}$ line with mice harboring a conditional oncogenic *Kras*^{G12D} mutant allele silenced by a floxed transcriptional STOP cassette (Lox-Stop-Lox or LSL) (20). The removal of the LSL cassette by Cre recombinase driven by the *Pdx1* gene promoter allows endogenous expression of Kras^{G12D} in pancreatic cell lineages (2). The resulting offspring that had received TM through their mother's milk 7 days after birth were killed at 3, 6 and 9 months of age and subjected to histological analysis to assess pancreatic pathology (Fig. 2A-F). Pancreata of *Pdx1-Cre*^{ER};*Kras*^{G12D};*mkk4*$^{+/lox}$ mice were mostly normal up to an age of 3 months, with apparent acinar-to-ductal metaplasia (ADM; ductal lesions containing acinar granules) detected in 1 of the 2 animals analyzed (Fig. 2A and G). As expected, mostly small focal low-grade 1A and 1B mPanINs were noted as the mice aged (Fig. 2B and G). Similar to that seen in the *Pdx1-Cre*;*Kras*^{G12D} strain (2, 3), these neoplastic ducts rarely progressed, with only one mouse displaying mPanIN-2 and PDAC at 9 months of age (Fig. 2C and G).

When Kras^{G12D} expression in the pancreas was combined with *mkk4* gene deletion, all the mice in the cohort displayed ADM and the majority exhibited mPanIN-1B as early as 3 months of age (Fig. 2D and G). The incidence of high-grade ductal lesions significantly increased with age, as evident by the detection of multiple mPanIN-2 and mPanIN-3 lesions with intense stromal reaction (stromal desmoplasia) in pancreata of older mice (Fig. 2E-G). Importantly, mPanIN-3 transitioning to carcinoma in situ occurred with complete penetrance after 6 months (Fig. 2G). An mPanIN score index was calculated based on observation by a blinded pathologist (R.F.T.M.) and weighted according to increasing lesion stage (*see*

Materials and Methods) to rigorously demonstrate the acceleration of disease progression caused by the absence of MKK4 (Fig. 2H).

Consistent with human PanINs, neoplastic ducts observed in *Pdx1-Cre^{ER};Kras^{G12D};mkk4$^{lox/lox}$* mice at all ages (3-9 months) expressed the ductal cell marker cytokeratin 19 (CK19) and produced mucin detected by Alcian Blue staining (Fig. 2I-N). CK19 positive carcinoma cells imbedded within the stroma were detected in pancreata of compound mutant mice at 9 months of age, indicative of invasive PDAC (Fig. 2K, arrow). Together, these results clearly established the functional requirement of MKK4 to suppress the oncogenic potential of activated Kras in pancreatic cells, a concept in line with high incidence of *mkk4* loss-of-function mutation associated with human PDAC (11, 12).

The absence of both MKK4 and MKK7 markedly accelerates the development of malignant ductal lesions caused by Kras^{G12D} expression

To determine whether MKK7 can, at least partially, compensate for the loss of MKK4 expression, we examined the effect of combining Kras^{G12D} expression with homozygous deletion of both *mkk4* and *mkk7* alleles. The *Pdx1-Cre^{ER};Kras^{G12D};mkk4$^{lox/lox}$;mkk7$^{lox/lox}$* mice were born at the expected Mendelian frequency and were given TM through their mother's milk 7 days after birth. By 10 weeks of age 2 animals in the cohort (n = 6) presented with jaundice and ascites. Based on this observation, all the animals were sacrificed for autopsy prior to becoming further distressed. No mice with other genotypes displayed any of these symptoms at any stage of the analysis. Representative images show that the pancreas of compound mutant mice at 10 weeks is as large as that of 9 months old control cohorts (*Pdx1-Cre^{ER};Kras^{G12D};mkk4$^{+/lox}$* and *Pdx1-Cre^{ER};Kras^{G12D};mkk4$^{lox/lox}$*) (Fig. 3A-C). These abnormally large and irregularly shaped pancreata were firm and fibrotic, occasionally obstructing the common bile duct and causing dilation of the gall bladder (Fig. 3C and D).

Histological analyses indicated that, by 10 weeks of age, all the mice (n = 6) presented widespread high grade 2 and 3 dysplastic ductal structures with intense stromal desmoplasia, resulting in almost the complete loss of normal acinar, ductal and islet tissues (Figures 2G and 3E). The mPanIN score index confirmed that the loss of both *mkk4* and *mkk7* drastically accelerated the progression of advanced-stage cancer (Fig. 2H). To verify that the dramatic increase in disease progression observed through compound *mkk4* and *mkk7* deletion was via the co-operative effect of losing both JNK activators, rather than through losing *mkk7*, we generated *Pdx1-Cre^{ER};Kras^{G12D};mkk7$^{lox/lox}$* mice. Of the 7 mice exposed to TM, all developed low grade mPanIN-1 lesions at 3 months of age and only 1 displayed mPanIN-2, but no invasive PDAC was observed (Fig. 2G). Hence, *mkk4* or *mkk7* deleted mice had a comparable mPanIN score index (Fig. 2H). This observation demonstrated that MKK4 and MKK7 functioned in a partially non-redundant manner within the transformed pancreas.

Consistent with a ductal phenotype, mPanINs observed in *Pdx1-Cre^{ER};Kras^{G12D};mkk4$^{lox/lox}$;mkk7$^{lox/lox}$* mice exposed to TM exhibited abundant mucin content detected by Alcian Blue staining and were positive for CK19 (Fig. 3F and G). Evidence of advanced PDAC is substantiated by nascent invasion of CK19 expressing cells beyond the basement membrane and into the stroma (Fig. 3G, arrow) and TFGβ1 positive

tumor cells migrating towards the gut (Fig. 3H, inset). Increased autocrine TFGβ1 expression caused by impaired JNK signaling in the context of Ras activation is predicted to contribute to promoting invasion and metastasis (25). Interestingly, neoplastic lesions expressing the pancreatic progenitor cell marker Pdx1 were deficient in MKK4 expression (Fig. 3I and J). This is consistent with the idea that PanINs can arise from de-differentiated acini that are reprogrammed into metaplastic structures expressing embryonic pancreatic and ductal markers. As expected, dual phosphorylation of JNK at Thr and Tyr residues in the activation loop was readily detectable in mPanINs observed in the pancreas of $Kras^{G12D}$ mice (Fig. 3K). This was absent in compound mutant mice expressing $Kras^{G12D}$ and lacking MKK4 and MKK7 (Fig. 3L and M). Together, these results clearly demonstrated that the blockade of JNK signaling interacted synergistically with the expression of activated Kras to promote invasive PDAC.

Molecular analyses of MKK4 and MKK7 deficient pancreatic tumors

The rapid progression of mPanINs caused by impaired JNK signaling provided us with an opportunity to analyze the status of clinically relevant molecular determinants associated with pancreatic cancer. In particular, Notch signaling is normally suppressed in the adult organ. However, this pathway has been identified as abnormally up-regulated in both human and murine PanIN lesions and may be critical for maintaining transformed cells in an un-differentiated state (2, 26-28). Activation of Notch signaling requires γ secretase-dependent cleavage of the Notch receptor. Using an antibody specific for the cleaved intracellular domain (ICD) of the Notch 1 receptor we found activated Notch 1 in mPanINs, but not in morphologically normal acini (Fig. 4A and B). Notch ICD labelling was especially strong in ductal adenocarcinoma (Fig. 4C). Likewise, ERK1/2 phosphorylation, indicative of ERK1/2 activation, was up-regulated in ductal lesions (Fig. 4D-F). The intensity of the signal increased as mPanINs progressed to more advanced stages, supporting the idea that ERK1/2 signaling provides growth and survival advantages to carcinoma cells. Finally, mPanINs displayed strong membranous and cytoplasmic accumulation of β-catenin (Fig. 4G and H). The redistribution of the protein from the membrane to the nucleus in cells associated with high grade PanINs/PDAC is indicative of transcriptional activation of the canonical Wnt signaling pathway during disease progression (Fig. 4I, arrow). Accordingly, although β-catenin can block the de-differentiation of acinar cells, this pathway has been associated with pancreatic tumor growth (29). At all stages, the lesions were distributed within a rich stromal component positive for αSMA staining (Fig. 4J-L). Consistent with concomitant inflammation associated with tumor growth, STAT3 phosphorylation was readily detected in mPanINs and in surrounding stromal cells (Fig. 4M-O).

To understand the mechanism underlying the synergistic interaction between *mkk4*/*mkk7* loss-of-function mutation and oncogenic Kras activation, we examined the possibility that JNK signaling controlled acinar cell reprogramming. Tissues were co-immunolabelled for amylase (acinar-specific) and CK19 (duct-specific) (Fig. 5). As expected, normal pancreata (*Pdx1-Cre^{ER};mkk4$^{+/lox}$*) are mostly formed of amylase-positive acinar tissue with very low level of CK19 labelling and little or no evidence of co-staining (Fig. 5A and B). In contrast, metaplastic structures consisting of acinar cells (green), ductal-like cells (red) and bi-phenotypic cells co-expressing both amylase and CK19 (yellow) were detected next to

normal acinar parenchyma in *Pdx1-Cre^{ER};Kras^{G12D};mkk4$^{+/lox}$* samples (Fig. 5C and D). Impaired JNK signaling drastically accelerated the appearance of acinar metaplasia, with no normal pancreatic tissue remaining in 10 weeks old *Pdx1-Cre^{ER};Kras^{G12D};mkk4$^{lox/lox}$;mkk7$^{lox/lox}$* animals (Fig. 5E and F). Quantitative analysis confirmed that the absence of MKK4 and MKK7 in the context of *Kras^{G12D}* expression resulted in a significant reduction in amylase level together with increased CK19 expression (Fig. 5G). The number of bi-phenotypic cells was similar to that seen with Kras^{G12D} alone, consistent with the idea that the loss of *mkk4* and *mkk7* expression facilitates the trans-differentiation of acinar cells into duct-like cells (Fig. 5G). Together, these studies genetically demonstrated that MKK4 and MKK7 were required to suppress Kras^{G12D}-induced reprogramming of acinar cells into pancreatic ductal lesions.

MKK4 and MKK7 are required for acinar regeneration

To establish a general role of MKK4 and MKK7 in controlling the fate of acinar cells undergoing de-differentiation, we tested the effect of *mkk4*/*mkk7* loss-of-function in a model of caerulein-induced inflammatory ductal metaplasia (Fig. 6). Unlike ADM associated with oncogenic Ras signaling, the reprogramming of acinar cells into duct-like cells induced by acute caerulein pancreatitis is a transient process necessary to regenerate the pancreas (30). *mkk4$^{lox/lox}$;mkk7$^{lox/lox}$* offspring carrying or not the *Pdx1-Cre^{ER}* transgene received TM through their mother's milk 7 days after birth. Five weeks later, the animals received 6 hourly intra-peritoneal injection of PBS or caerulein on 2 days separated by a 48 hours period. Mice were sacrificed 2 or 7 days after the last injection. PBS treated control animals were sacrificed 7 days after the last injection.

Consistent with the ability of the pancreas to regenerate, caerulein-induced pancreatitis was fully reversible in *mkk4$^{lox/lox}$;mkk7$^{lox/lox}$* animals (Fig. 6A-C). This was corroborated by the transient decreased expression of amylase concomitant with increased CK19 expression (Fig. 6G-I and M). Acinar trans-differentiation into duct-like cells is supported by increased number of cells expressing both amylase and CK19 in samples collected 2 days after caerulein injection (Fig. 6M). By 7 days, amylase and CK19 were redistributed in a fashion similar to that seen with PBS treatment (Fig. 6G, I and M). Exocrine damage with evidence of acinar cell reprogramming was visible in pancreata of *Pdx1-Cre^{ER};mkk4$^{lox/lox}$;mkk7$^{lox/lox}$* animals 2 days after being exposed to caerulein (Fig. 6E, K and M). However, unlike the control cohort (*mkk4$^{lox/lox}$;mkk7$^{lox/lox}$*), acinar regeneration was not completed by 7 days after caerulein treatment (Fig. 6F). Accordingly, decreased amylase expression together with elevated CK19 expression persisted in MKK4/MKK7-deficient pancreata (Fig. 6L and M). This resulted in the replacement of normal exocrine tissue with ductal structures consisting of amylase- and CK19-positive cells embedded in a reactive stroma (Fig. 6F and L). Normal amylase-expressing acinar cells were rare.

To understand why impaired JNK signaling blocked acinar cell reprogramming, thereby preventing pancreatic regeneration, we compared the molecular signature of acute pancreatitis in *mkk4$^{lox/lox}$;mkk7$^{lox/lox}$* and *Pdx1-Cre^{ER};mkk4$^{lox/lox}$;mkk7$^{lox/lox}$* cohorts (Fig. 7). In response to damage, acinar cells reactivate transiently the expression of factors normally expressed by progenitor cells during embryonic development (30). Accordingly, 2

days after caerulein treatment, a slight, but nonetheless noticeable, increase in *Pdx1* gene expression was detected in the exocrine compartment of both control and *mkk4*/*mkk7* deleted animals (compare Fig. 7A and B with 7D and E). The ductal phenotype acquired by the de-differentiated acinar cells following damage is supported by elevated expression of Sex-determining region Y (SRY) box 9 (Sox9) (compare Fig. 7G and H with J and K). Normal expression pattern of these progenitor and ductal markers was re-established 7 days later in control samples, when regeneration was complete (Fig. 7C and I). In contrast, expression of Pdx1 and Sox9 remained elevated in duct-like structures observed in pancreata of *mkk4*/*mkk7* deleted animals (Fig. 7F and L). Additionally, consistent with the requirement of β-catenin for efficient acinar regeneration (29), $mkk4^{lox/lox};mkk7^{lox/lox}$ mice exhibited a transient up-regulation of β-catenin (Fig. 7M-O). In contrast, β-catenin accumulation was still visible in pancreata of $Pdx1\text{-}Cre^{ER};mkk4^{lox/lox};mkk7^{lox/lox}$ animals 7 days after exposure to caerulein (Fig. 7P-R). Together these data genetically demonstrated that MKK4/MKK7-deficient acinar cells were unable to regenerate from a de-differentiated state.

Discussion

To rigorously establish a causal relationship between JNK signaling and Kras-dependent initiation of PDAC, we examined the effect of loss-of-function mutation of *mkk4* together with or without loss of *mkk7* expression in mice expressing physiological level of oncogenic Kras in the pancreas. Our results unequivocally demonstrate that impaired JNK signaling sensitizes the pancreas to $Kras^{G12D}$-induced mPanIN formation, with strong genetic evidence that $Kras^{G12D}$ combined with compound MKK4/MKK7 deficiency is sufficient to produce advanced malignant disease. These experimental data are consistent with genomic analyses of human carcinoma which predicted the tumor suppressive function of MKK4 in pancreatic cancer (11, 12, 31, 32). Accordingly, a potential correlation was proposed between decreased survival of patients with wide spread disease and loss of MKK4 protein expression in metastatic cancer tissues (33). Recurrent deletion mutation in the *mkk4* locus was also identified in human prostate and breast cancers (34, 35). Consistently, genetic analyses in mice have demonstrated that JNK isoforms act as suppressors of prostate and mammary tumor development (22, 36-38).

Unlike *mkk4*, the *mkk7* locus is rarely found mutated in human cancer genomes. This is intriguing considering that *mkk7* gene deletion cooperated with the loss of *mkk4* to produce advanced PDAC. Likewise, invasive adenocarcinoma of the prostate was only detected following the functional inactivation of both, *mkk4* and *mkk7* genes in the Δ*Pten* mouse model (22). Furthermore, like MKK4, MKK7 suppressed $Kras^{G12D}$-driven lung carcinoma (39, 40) and NeuT-driven mammary cancer (40). In light of these findings, we can speculate that MKK7 may not be selected against by tumors because it serves temporally as an essential regulator of adult acinar cell proliferation and survival in the premalignant stage of the disease. This hypothesis rests on previous genetic evidence that MKK7 and JNK are required for protecting murine fibroblasts from premature senescence (41, 42). Oncogenic-induced senescence (OIS), first discovered in primary human or rodent cells expressing oncogenic *ras* (43), is now widely recognized as a bona fide negative regulator of tumorigenesis and is a feature of low grade PanINs carrying *Kras* oncogene (9, 44). Alternatively, inactivation of the *mkk7* locus may occur in cancer via epigenetic

modifications, rather than genetic mutations. This possibility highlights the limitation of unbiased genome-wide analyses of human tumors to identify clinically relevant molecular determinants of tumorigenesis.

In recent years, the remodelling of the microenvironment via stimulation of inflammatory cells and cytokine production has been shown to be critically important in supporting, not only the *de novo* development of tumors, but also many aspects of cancer progression (45). In particular, the importance of the immune system in PDAC development was highlighted by the demonstration that the resistance of adult mice to oncogenic *Kras* expression in acinar cells can be overcome by pancreatitis, one of the highest risk factor for PDAC development in human (7-10). Exocrine damage associated with pancreatic inflammation causes the transient de-differentiation of pre-existing acini into duct-like structures to restore homeostasis (30, 46). We found that acinar regeneration requires a functional JNK signaling pathway. Therefore, it is possible that pancreas-specific *mkk4* and *mkk7* gene deletion in the context of endogenous $Kras^{G12D}$ expression enhances PDAC development by facilitating acinar metaplasia, giving rise to mPanIN formation. Accordingly, ADM is one of the earliest morphological alterations observed in the pancreas of $Kras^{G12D}$ *mkk4/mkk7* deleted mouse model and is a feature a human PDAC. Overall, the idea that JNK signalling controls cellular plasticity may have far reaching implications in cancer biology, as compound JNK deficiency in ΔPten mice was also shown to correlate with an expansion of the immature prostate cell population with increased survival and self renewing capabilities (22).

Recent studies have started to shed some light into the molecular link between inflammation and PDAC initiation (47). In particular, the loss of STAT3 signaling in the $Kras^{G12D}$ mouse model was shown to significantly delay the development of mPanIN lesions (48, 49). This correlated with reduced inflammatory cell infiltration and cytokine expression, including interleukin 6 (IL6). STAT3 activation by IL6 through a positive feedback loop mechanism promotes the survival and proliferation of transformed cells and support metaplasia-associated inflammation (48, 49). Interestingly, compound deficiency of JNK1 plus JNK2 in hepatocytes caused an up-regulation of IL6-dependent stimulation of STAT3 in the liver of mice exposed to the carcinogen diethylnitrosamine-phenobarbital (DEN) (50). This effect was associated with increased tumor size in the model of DEN-induced hepatocellular carcinoma (HCC). Likewise, the pancreas of $Kras^{G12D}$ mice that lacked expression of MKK4/MKK7 displayed strong phosphorylation of STAT3 in mPanINs and in surrounding stromal cells. Therefore, it is tempting to propose that one of the mechanisms by which MKK4/MKK7 deficiency synergizes with oncogenic Kras is by enhancing signaling through the IL6/STAT3 axis, thereby creating a permissive environment for metaplastic changes to occur. This may involve the re-expression of embryonic progenitor and ductal markers, such as Pdx1 and Sox9, in the acinar compartment of the adult pancreas. Accordingly, inhibition of Sox9 in acinar cells suppressed $Kras^{G12D}$-induced formation of ADM and mPanINs, while forced expression of Sox9 facilitated the development of ADM and mPanINs (51, 52). The molecular basis for the *in vivo* cooperative interaction of activated Kras and MKK4/MKK7 deficiency in ADM will remain an important issue to address.

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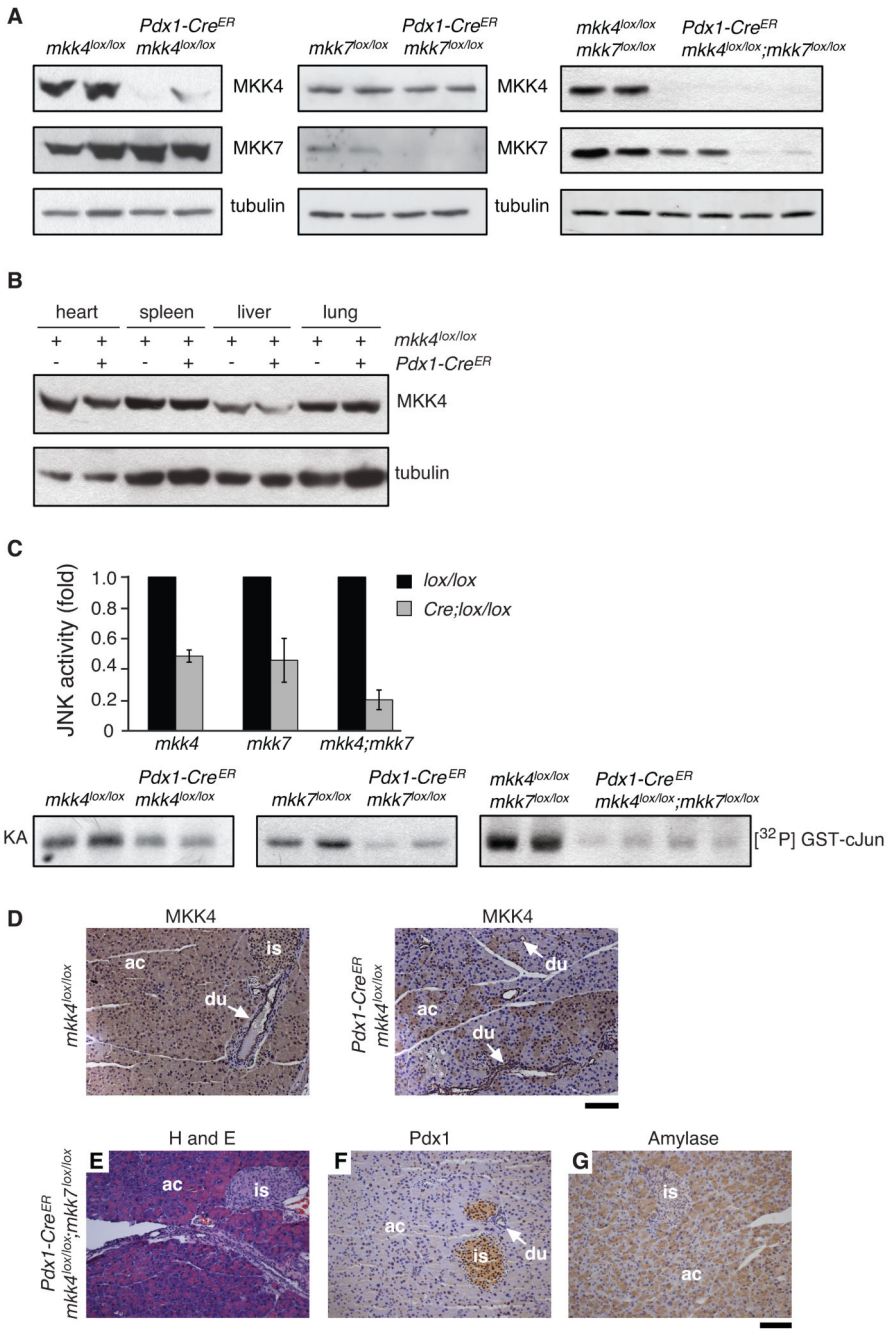

Figure 1. MKK4 and MKK7 are not required for pancreatic homeostasis.

A and **B**, Immunoblot analyses of MKK4 and MKK7 expression in organs derived from 6 weeks old *mkk4*$^{lox/lox}$, *mkk7*$^{lox/lox}$ and compound *mkk4*$^{lox/lox}$;*mkk7*$^{lox/lox}$ mice carrying or not the *Pdx1-Cre*^{ER} transgene and exposed to TM at 7 days after birth. The images represent distinct animals for each genotype. **C,** Endogenous JNK activity in pancreatic extracts was measured by protein kinase assay (KA). Radioactivity incorporated in GST-cJun was quantified by phosphoImager. The data correspond to the mean ± SD (N = 2). The autoradiography represents distinct animals for each genotype. **D,** MKK4 immunostaining

of pancreata extracted from 6 weeks old animals reveals that Cre-mediated recombination of the mutant allele occurs in a mosaic fashion. **E-G**, Compound deletion of *mkk4* and *mkk7* does not disrupt the architecture of the pancreas in 6 weeks old mice as determined by haematoxylin and eosin (H and E) staining (**E**), Pdx1 immunostaining of islets (**F**) and amylase immunostaining of acinar cells (**G**). ac = acinar cells; du = ducts; is = islets. Scale bars represent 200 μm.

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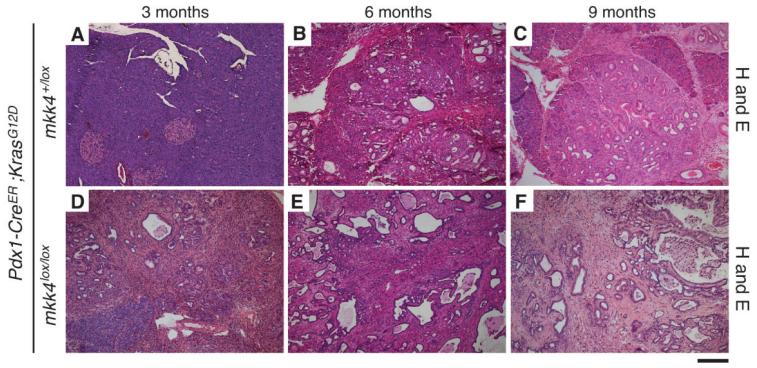
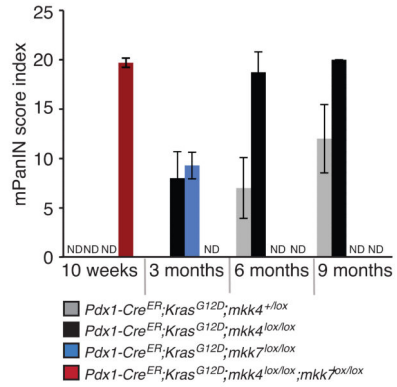


G

		ADM	PanIN-1A	PanIN-1B	PanIN-2	PDAC
Pdx1-Cre^{ER};mkk4$^{lox/lox}$	9 months	0/4	0/4	0/4	0/4	0/4
Pdx1-Cre^{ER};Kras^{G12D}; mkk4$^{+/lox}$	3 months	1/2	0/2	0/2	0/2	0/2
	6 months	3/3	3/3	3/3	1/3	0/3
	9 months	3/3	3/3	3/3	1/3	1/3
Pdx1-Cre^{ER};Kras^{G12D}; mkk4$^{lox/lox}$	3 months	3/3	0/3	2/3	1/3	0/3 (2[a])
	6 months	0/4	1/4	4/4	3/4	4/4 (1[b])
	9 months	0/2	0/2	2/2	2/2	2/2 (2[c])
Pdx1-Cre^{ER};Kras^{G12D}; mkk7$^{lox/lox}$	3 months	5/7	6/7	7/7	1/7	0/7
Pdx1-Cre^{ER};Kras^{G12D}; mkk4$^{lox/lox}$;mkk7$^{lox/lox}$	10 weeks	1/6	0/6	6/6	6/6	6/6 (2[d])

a PanIN associated with chronic pancreatitis
b Invasive tumour penetrating peritoneal cavity
c PDAC associated with ovarian-like stroma
d Jaundice and distended gall bladder

H



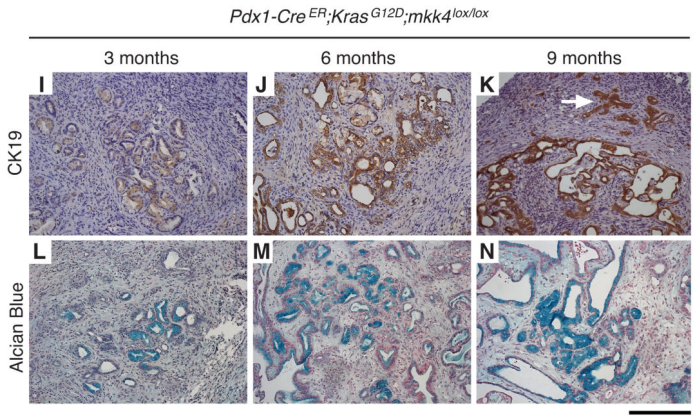


Figure 2. MKK4 deficiency accelerates Kras^{G12D}-induced mPanIN progression.

A-F, H and E staining of pancreata derived from mice exposed to TM. Expression of Kras^{G12D} alone induces ADM and the formation of low-grade mPanIN lesions that increase with severity as mice age (compare **B** and **C**). The simultaneous deletion of *mkk4* greatly accelerates mPanIN formation as evident within 3 months of age (**D**). By 6 months of age all mice had developed adenocarcinoma (**E** and **F**). **G,** Table indicating the number of mice per genotype presenting with specific pancreatic pathology. **H,** mPanIN score index in pancreata of mice exposed to TM were calculated as described in *Materials and Methods*. A score of 0

indicate normal pancreas pathology while 20 indicate that all mice present with PDAC. The data correspond to the mean ± SD. ND = not determined. **I-N,** PanIN lesions and adenocarcinoma observed in pancreata of *Pdx1-Cre^{ER};Kras^{G12D};mkk4$^{lox/lox}$* mice exposed to TM display a ductal phenotype as determined by immunohistochemical analysis for CK19 expression (**I-K**) and mucin production (**L-N**). The arrow indicates CK19 expressing epithelial tumor cells invading into the stromal region. Scale bar represents 200 μm.

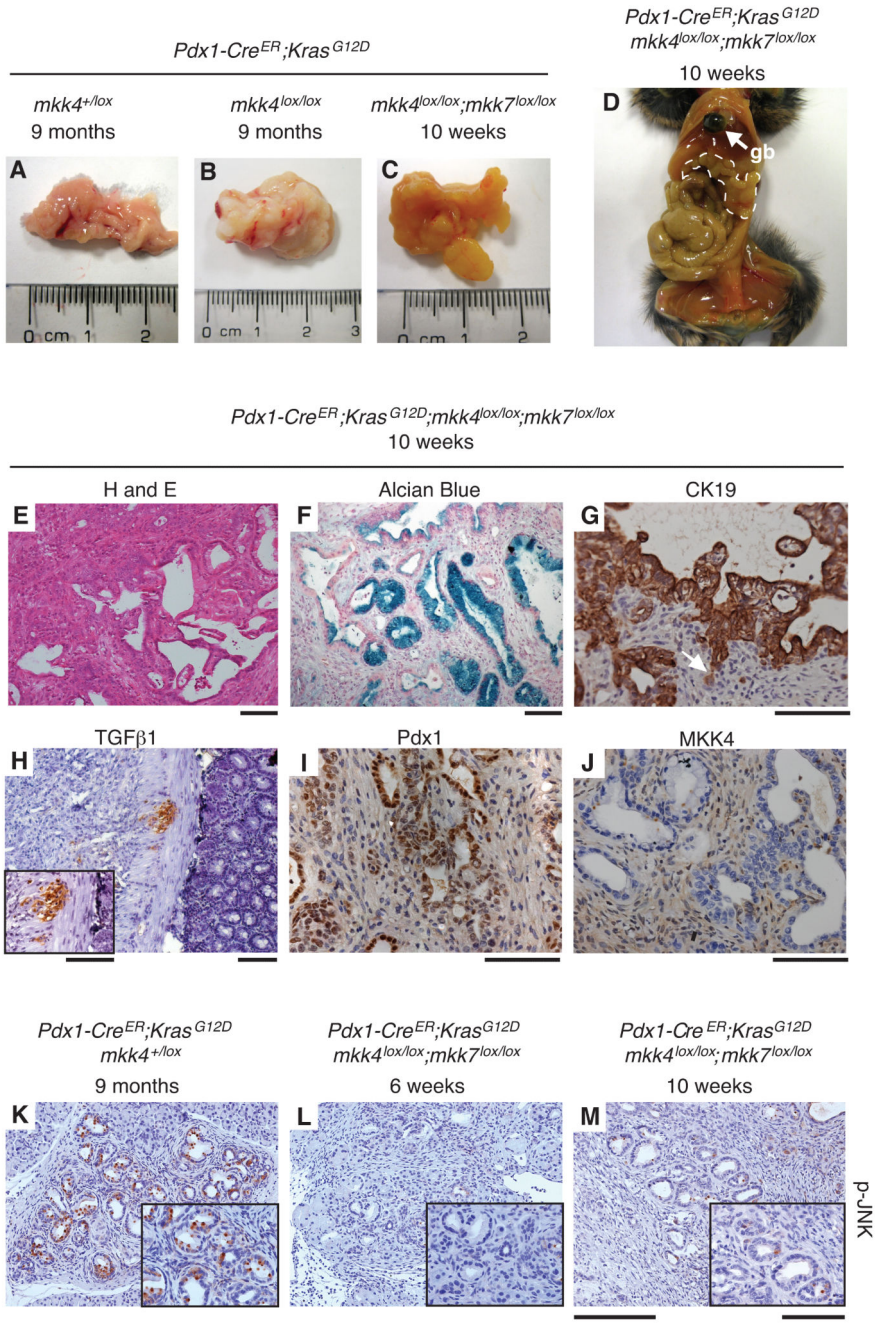


Figure 3. MKK4/MKK7-deficient mice expressing Kras^{G12D} develop PDAC with high penetrance and short latency.

A-C, Representative images of whole pancreata from 9 months old and 10 weeks old control and experimental animals exposed to TM. (**D**) Photograph of a pancreatic adenocarcinoma obstructing the common bile duct and causing dilatation of the gall bladder (gb) and jaundice in the abdominal skin. Dashed line denotes the tumors. (**E**) H and E staining show well-differentiated ductal adenocarcinoma with abundant stoma in Kras^{G12D} expressing *mkk4/mkk7* deleted mice at 10 weeks of age. All mice of this genotype (6 out of 6) rapidly

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developed PDAC within 10 weeks of age (see also Fig. 2G and H for quantification of PanIN lesions). **F-M,** Immunohistochemical analyses confirm the mucin-producing (Alcian Blue, **F**) and ductal phenotype (CK19, **G**) of the tumors. The arrow indicates nascent invasion of CK19 expressing tumor cells into the stroma. Inset (**H**) shows TGFβ1-positive tumor cells migrating toward the gut. Additionally, the neoplastic ductal structures re-express the progenitor marker Pdx1 (**I**), but lack MKK4 (**J**), while MKK4 is expressed within the stroma compartment (**J**). p-JNK was readily detectable in spontaneously formed mPanINs in 9 months old $Kras^{G12D}$ expressing mice (**K**), but was absent in lesions displayed by 6 and 10 weeks old $Kras^{G12D}$ expressing *mkk4/mkk7* deleted animals (**L, M**). Scale bars represent 200 μm or 100 μm for inset in **H**.

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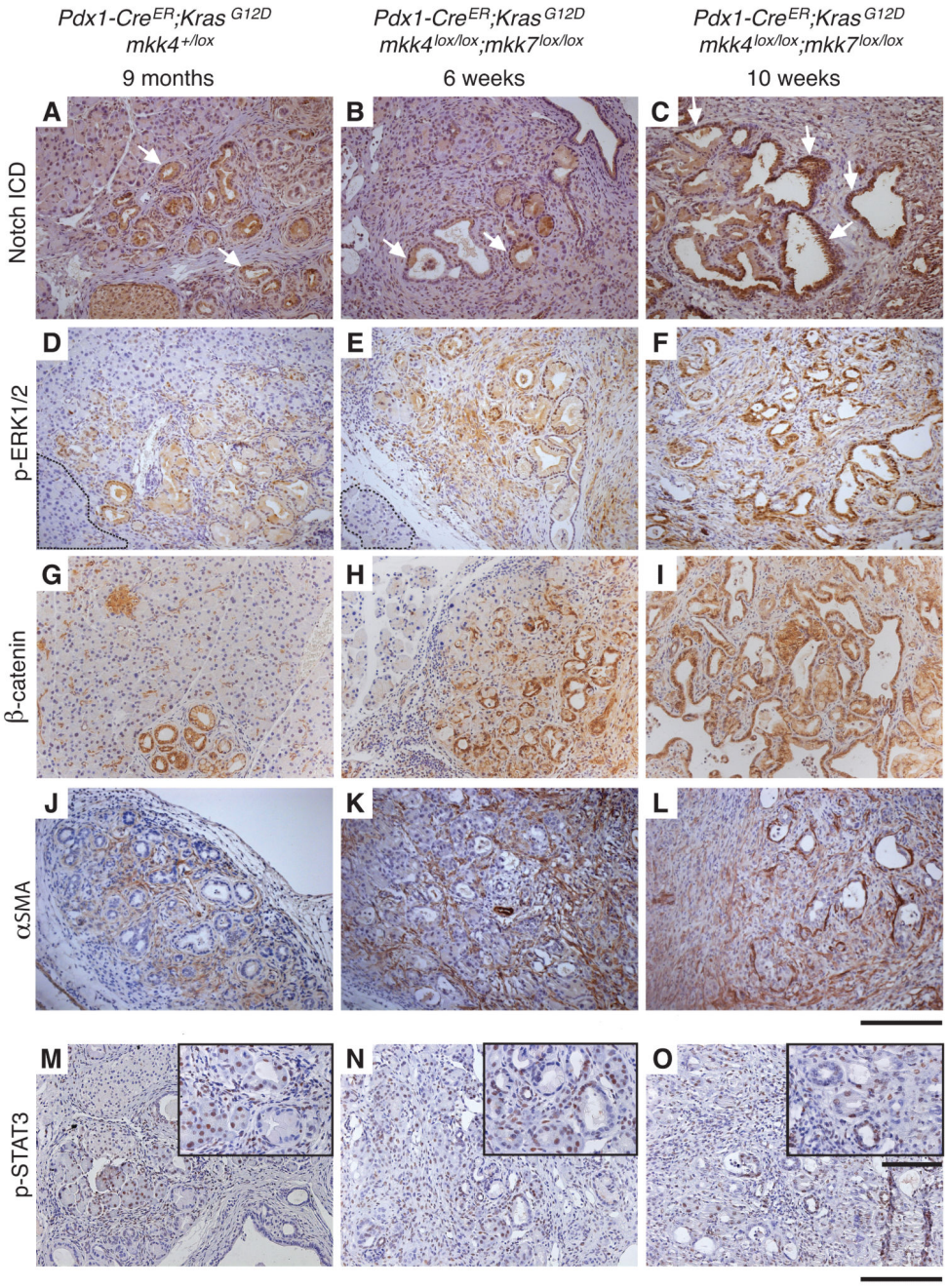

Figure 4. Notch, ERK1/2, β-catenin and STAT3 signaling pathways are up-regulated in pancreatic adenocarcinoma.

Immunohistochemical analyses demonstrate that nuclear Notch ICD (arrows) in mPanIN lesions (**A** and **B**) is substantially up-regulated in adenocarcinoma (**C**). The level of p-ERK1/2 is low in normal acinar parenchyma (highlighted areas in **D** and **E**), but elevated in mPanIN lesions and highly increased in adenocarcinoma (**F**). β-catenin progressively accumulates through the transition from mPanIN to PDAC formation (**G-I**). Tumors are surrounded by abundant stroma (**J-L**). STAT3 phosphorylation is detected in epithelial cells

of spontaneously formed mPanINs and in surrounding stromal cells (**M-O**). Scale bars represent 200 μm or 100 μm for insets in **M-O**.

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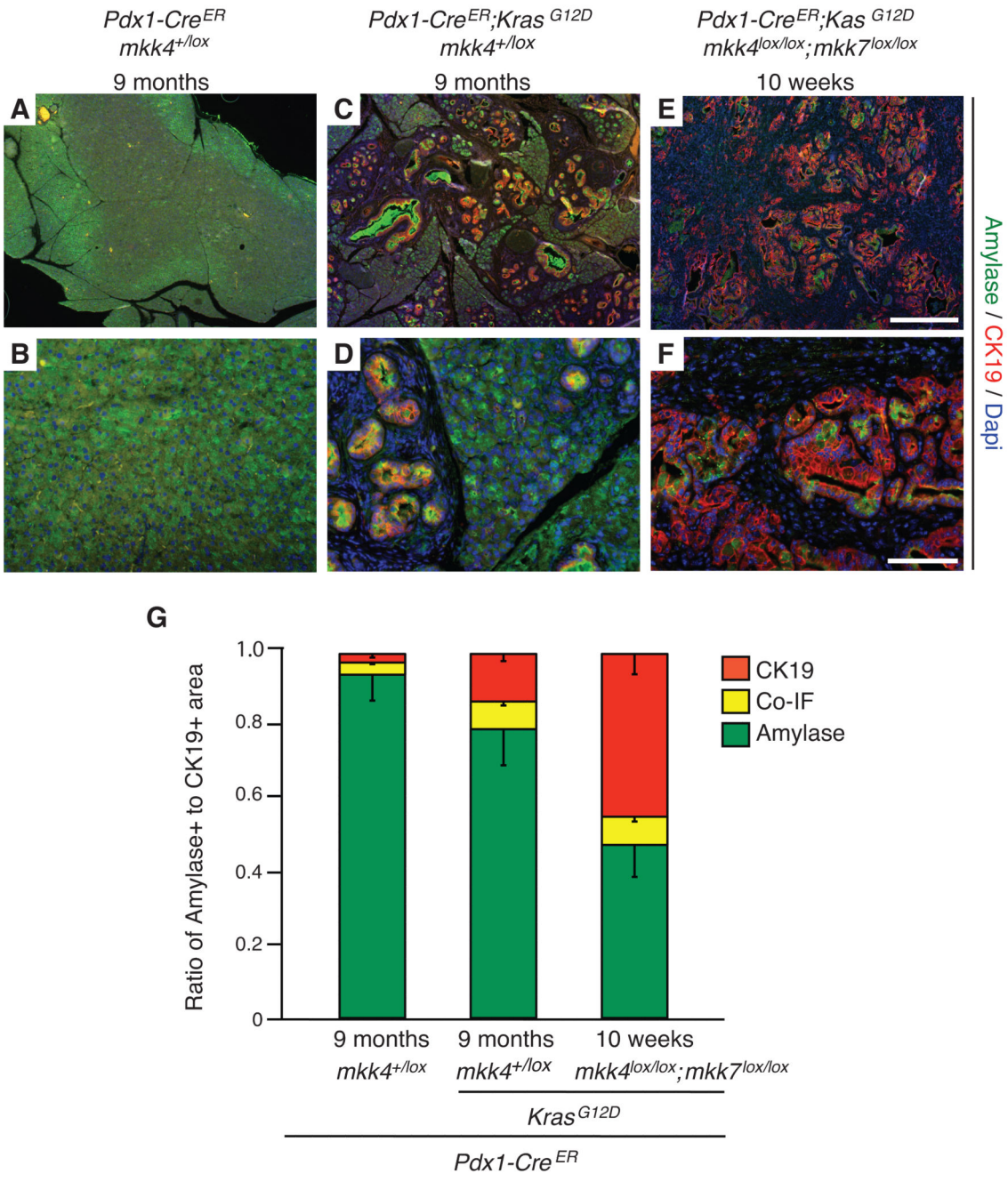

Figure 5. The loss of *mkk4* and *mkk7* accelerates the appearance of CK19 positive neoplastic ductal structures.

A-F, Immunofluorescence staining for amylase positive acinar cells (green) and ductal expressing CK19 cells (red). Normal pancreata predominantly comprise of amylase positive acinar cells (**A** and **B**). Expression of oncogenic Kras leads to the formation of abnormal structures consisting of both amylase and CK19 positive cells (**C** and **D**). The loss of *mkk4*/*mkk7* in the context of $Kras^{G12D}$ leads to the formation of predominant amylase and CK19 positive structures with a ductal morphology (**E** and **F**). Scale bars represent 500 μm (**A**, **C** and **E**) or 100 μm (**B**, **D** and **F**). **G,** Ratio of amylase, CK19 and amylase-CK19 co-

immunofluorescence (co-IF) areas for each of the representative genotypes. N ≥ 3. Error bars represent SEM.

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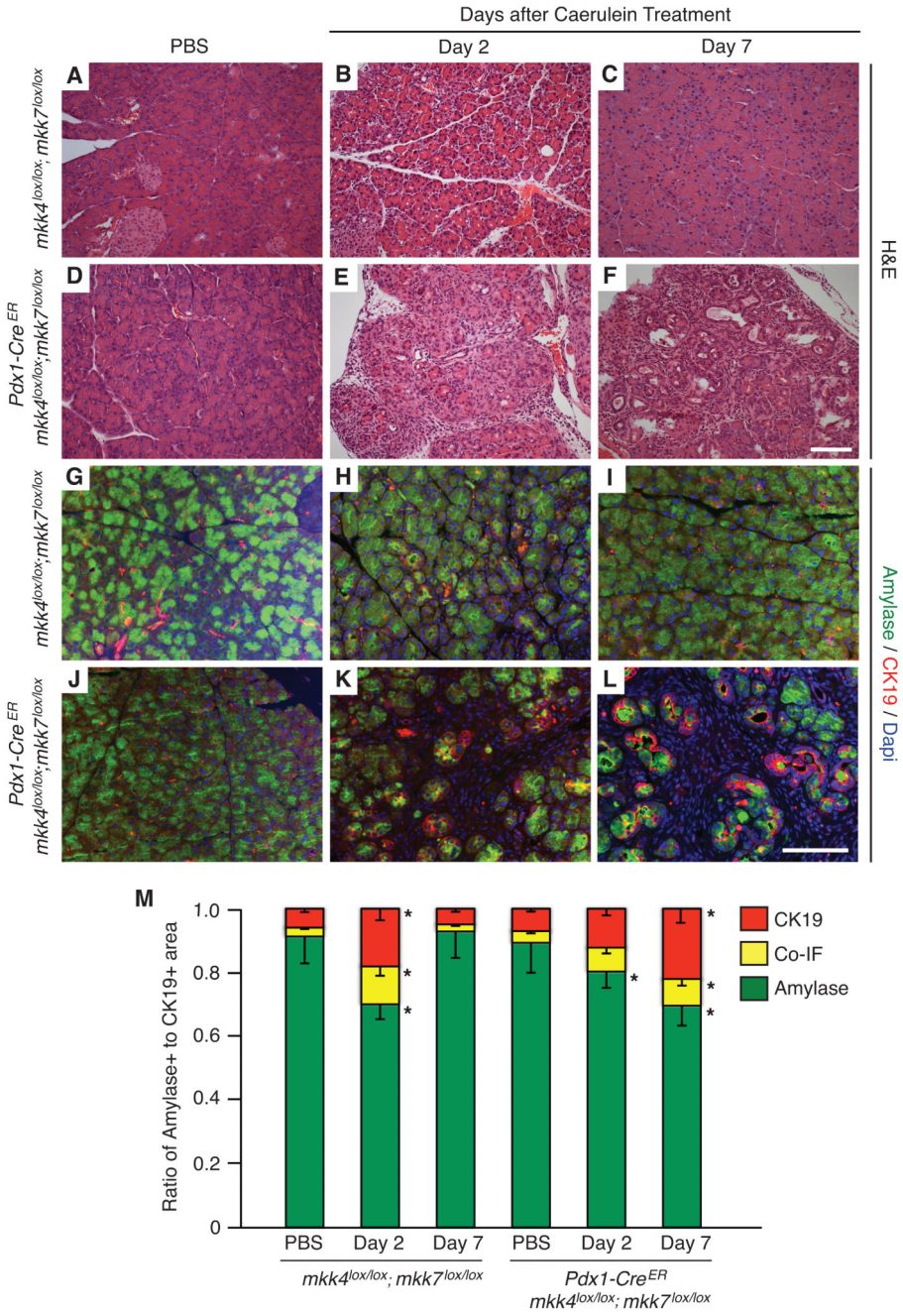

Figure 6. JNK signaling controls acinar cell reprogramming after induction of acute pancreatitis.

Gene deletion was induced at day 7 through TM administered by oral gavage of lactating dams. At 5 weeks of age, mice were exposed to caerulein and sacrificed 2 or 7 days later. PBS treated control animals were sacrificed 7 days after the last set of injections. **A-F,** H and E staining of pancreata from control (*mkk4*$^{lox/lox}$;*mkk7*$^{lox/lox}$) and *mkk4/mkk7* deleted (*Pdx1-Cre*^{ER};*mkk4*$^{lox/lox}$;*mkk7*$^{lox/lox}$) mice. The deletion of *mkk4/mkk7* suppresses acinar regeneration (compare **C** and **F**). **G-L,** Immunofluorescence staining for amylase positive acinar cells (green) and ductal expressing CK19 cells (red). Both cohorts display sign of

acute pancreatitis 2 days after exposure to caerulein as evident by the replacement of normal amylase positive exocrine parenchyma (**G** and **J**) with ductal structures co-expressing amylase and CK19 (**H** and **K**). By 7 days after treatment, amylase and CK19 expression in control pancreata was redistributed in a fashion similar to that seen with PBS-treated animals (compare **G** and **I**). In contrast, compound mutant mice display predominantly amylase and CK19 positive ductal structures with rare normal amylase positive acini (**L**). Scale bars represent 100 μm. **M,** Ratio of amylase, CK19 and amylase-CK19 co-immunofluorescence (co-IF) for each of the representative genotypes. N ≥ 3. Error bars represent SEM. *$P < 0.05$ indicates a significant difference between mice treated or not with caerulein.

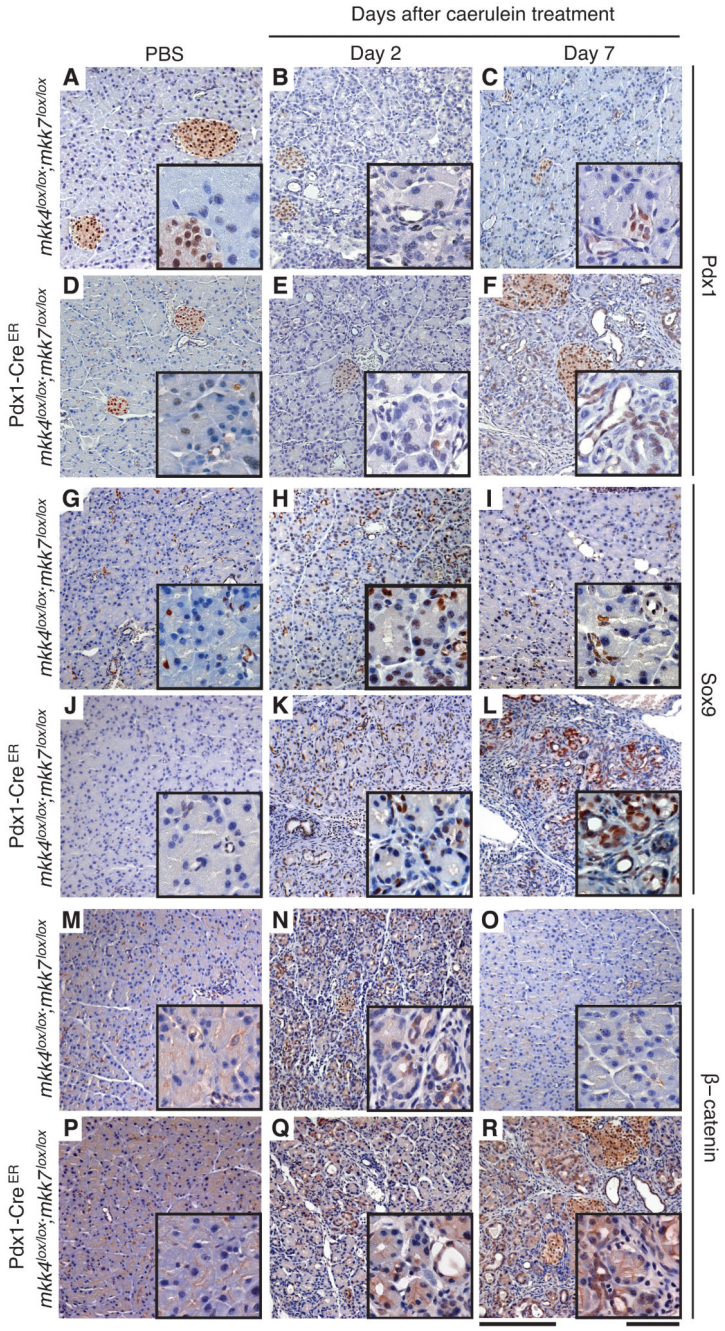

Figure 7. The loss of *mkk4* and *mkk7* locks damaged exocrine cells in a permanently de-differentiated state.

Immunohistochemistry with antibodies directed against Pdx1 (**A**-**F**), Sox9 (**G**-**L**) and β-catenin (**M**-**R**) in pancreatic tissues obtained from mice of the indicated genotype after caerulin treatment. Caerulein exposure causes a transient up-regulation of Pdx1, Sox9 and β-catenin in control mice (*mkk4*$^{lox/lox}$;*mkk7*$^{lox/lox}$). In contrast, 7 days after caerulein administration, duct-like structures expressing Pdx1, Sox9 and β-catenin persist in pancreata

derived from *Pdx1-Cre*^{ER};*mkk4*$^{lox/lox}$;*mkk7*$^{lox/lox}$ animals. Scale bars represent 200 μm (low magnification) or 50 μm (insets).

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