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## Canonical Wnt signaling is required for pancreatic carcinogenesis

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### Abstract

Wnt ligand expression and activation of the Wnt/ $\beta$ -catenin pathway have been associated with pancreatic ductal adenocarcinoma, but whether Wnt activity is required for the development of pancreatic cancer has remained unclear. Here we report the results of three different approaches to inhibit the Wnt/ $\beta$ -catenin pathway in an established transgenic mouse model of pancreatic cancer. First, we found that  $\beta$ -catenin null cells were incapable of undergoing acinar to ductal metaplasia, a process associated with development of premalignant PanIN lesions. Second, we addressed the specific role of ligand-mediated Wnt signaling through inducible expression of Dkk1, an endogenous secreted inhibitor of the canonical Wnt pathway. Lastly, we targeted the Wnt pathway with OMP-18R5, a therapeutic antibody that interacts with multiple Frizzled receptors. Together, these approaches demonstrated that ligand-mediated activation of the Wnt/ $\beta$ -catenin pathway is required to initiate pancreatic cancer. Moreover, they establish that Wnt signaling is also critical for progression of pancreatic cancer, a finding with potential therapeutic implications.

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## Keywords

Genetically engineered mouse model; Kras; MAPK; pancreatic cancer; Wnt

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## Introduction

Pancreatic ductal adenocarcinoma (PDA), the most common form of pancreatic cancer, is one of the deadliest human malignancies with an overall 5-year survival rate of less than 5% (1). There is a dire need for improving our understanding of the biology of pancreatic cancer in order to develop effective therapeutic options.

PDA is almost universally associated with the presence of a mutant, constitutively active form of Kras – most frequently the glycine to aspartic acid substitution Kras<sup>G12D</sup> – a small GTPase protein that is involved in signal transduction downstream of receptor tyrosine kinases [for review, see (2)]. Progression to PDA appears to occur through ductal precursor lesions, most commonly Pancreatic Intraepithelial Neoplasia (PanIN), that accrue morphological and molecular atypia. PDA is characterized by genomic instability, and a large number of mutations and chromosomal abnormalities are found in each individual tumor (3). Notwithstanding the complexity of their genome, most human PDAs display mutations in 12 core signaling pathways including Wnt and other embryonic signaling pathways (3). Genomic studies support previous work indicating inappropriate activation of Hedgehog, Notch and Wnt signaling in PDA [for review, see (4)]. While the functional role of Hedgehog and Notch signaling has been studied in depth, less is known about the function of Wnt signaling during disease progression.

The Wnt signaling pathway is complex and includes canonical and non-canonical branches [for review, see (5)]. For the purpose of this study, we focused our analysis to canonical Wnt signaling. The canonical pathway is activated by Wnt family ligands binding to Frizzled/ LRP receptor complexes. The cascade of events that ensues prevents  $\beta$ -catenin degradation within the cytoplasm and allows its stabilization and nuclear translocation. In the nucleus,  $\beta$ -catenin binds to transcriptional factors of the Tcf/Lef family to form a transcriptional activator (6). Both inhibitory components of the pathway, such as Axin 2, and transcriptional co-activators, such as Tcf1 and Lef1, are also target genes, contributing to the intricate feedback mechanisms that control pathway activity.

Mice with Cre dependent, conditional expression of oncogenic Kras<sup>G12D</sup> in the pancreas (hereby referred to as KC mice) develop PanIN lesions that progress to PDA, closely mimicking the human disease (7). Since these animals develop tumors in a step-wise manner in the context of an intact microenvironment, they have emerged as a relevant model for basic discovery as well as for preclinical studies [for review, see (8)]. KC mice also recapitulate the aberrant re-activation of embryonic signaling pathways, including Wnt, observed in the human disease (9). Thus, they constitute a valid model to study the functional requirement for Wnt signaling during pancreatic carcinogenesis.

Previous functional studies addressing Wnt signaling in PDA have been mainly based on enforced aberrant  $\beta$ -catenin activity using a dominant-active, degradation-resistant allele. When combined with mutant Kras, stabilized  $\beta$ -catenin prevents the formation of PanINs and PDA and instead leads to the development of tumors reminiscent of human intraductal tubular tumors (10), a rare form of pancreatic cancer unrelated to PDA. Thus, high-level activation of Wnt signaling is incompatible with PDA formation. On the other hand, accumulation of  $\beta$ -catenin and activation of Wnt target genes are observed in PanINs and PDA, (4, 9, 11, 12) and  $\beta$ -catenin functionally supports maintenance of PDA cell proliferation and tumor forming capacity in xenograft models (9, 13). This paradox

compelled us to explore whether activation of the Wnt/ $\beta$ -catenin pathway is required during pancreatic carcinogenesis, and what role it plays during different stages of disease progression.

We used three complementary approaches to block Wnt/ $\beta$ -catenin activation in the KC mouse model. *First*, we genetically inactivated  $\beta$ -catenin, a key pathway component. *Second*, we used a tetracycline-regulated system to overexpress Dkk1, a secreted protein that has been shown to specifically and effectively inhibit canonical-Wnt pathway activation (14, 15). *Third*, we took a therapeutically relevant approach by using OMP-18R5, a monoclonal antibody that inhibits Wnt signaling by binding Frizzled (Fzd) receptors (16).

## Materials and Methods

### Mouse strains

The mice were housed in specific pathogen-free facilities at the University of Michigan Comprehensive Cancer Center, or at the University of California, San Francisco. All the studies were conducted in compliance with the respective Institutional Committees on Use and Care of Animals guidelines. Detailed strain information is included in the Supplemental Methods.

### Cell lines

Primary mouse pancreatic cancer cell lines 65671, 4292, 9805 and primary human pancreatic cancer cell lines UM2, 1319, UM18 and UM19 were used in this study. 65671 was derived from the tumor of a KPC mouse (7) in pure FVBN genetic background. Additionally, an E2F-luciferase reporter allele was carried by that mouse, thus the tumor cells are labeled with luciferase. The cell line was derived in 2008, and genotyped for recombination of the conditional alleles to exclude contamination by non-epithelial cells; genotyping was repeated in 2012. 4292 and 9805 were derived from iKras\*<sup>p53</sup>\* tumors (17) and genotyped for recombination of the conditional alleles. UM2, 1319, UM18 and UM19 were derived from patients with confirmed diagnosis of pancreatic adenocarcinoma (University of Michigan) (18). The cell lines were passaged for less than 6 months and genotyped for mutant Kras to confirm its epithelial nature in 2012.

### Doxycycline treatment

Doxycycline was administered in the drinking water, at a concentration of 0.2g/L in a solution of 5% sucrose, and replaced every 3 to 4 days.

### Tamoxifen treatment

Tamoxifen was administered in 5 consecutive daily intraperitoneal injections of 1 mg. Mice were sacrificed for *in vitro* studies 2 weeks later.

### Three Dimensional Acinar Cell Culture

The 3D culture of Elastase-Cre;  $\beta$ -catenin<sup>f/f</sup> pancreatic acinar cells was prepared in a collagen matrix, as previously described (19); the culture media was supplemented with 100 ng/ml TGF- $\alpha$  to induce acinar-ductal metaplasia. KC or KDC mice were treated with doxycycline 3 days before harvest for 3D culture (also see Supplementary Material). The percentage of duct-like structures in 5 wells for each group was counted at day 3 of 3D culture. A two-tailed unpaired t test was used for statistical analysis.

### **OMP-18R5 treatment**

Monoclonal antibody OMP-18R5 was provided by Oncomed Pharmaceuticals (Redwood City, CA). OMP-18R5 was isolated from the MorphoSys HuCAL GOLD library. KC mice were treated with OMP-18R5 (10mg/Kg, twice/week) or PBS by intraperitoneal injection for 2 months before sacrificed for study. Primary mouse pancreatic cancer cell line 65671 and human pancreatic cancer cell line UM2 were treated with OMP-18R5 at 10 $\mu$ g/ml and 20  $\mu$ g/ml respectively in culture.

### **Recombinant Dkk1 treatment**

The mouse pancreatic cancer cell line 65671 and the human pancreatic cancer cell line UM2 were treated with recombinant mouse or human Dkk-1, respectively (500 ng/ml).

### **Histopathological analysis**

Histopathological analysis was conducted by a pathologist (W.Y.) on de-identified slides. Five images (20 $\times$  objective) were taken in standardized positions (as to cover the whole section) for each slide. A minimum of 50 total acinar or ductal clusters were counted from at least three independent animals for each group. Each cluster counted was classified as normal (nl), ADM, PanIN1A, 1B, 2 or 3 based on the classification consensus (20). The data was expressed as percentage of total counted clusters. Error bars represent SEM.

### **Immunohistochemistry and immunofluorescence**

Immunohistochemistry and immunofluorescence were performed as previously described (21). A list of antibodies is included in the Supplemental Methods, Table S1. Images were taken with an Olympus BX-51 microscope, and Olympus DP71 digital camera, and DP Controller software. The immunofluorescent images were acquired using an Olympus IX-71 confocal microscope and FluoView FV500/IX software.

### **Proliferation analysis**

The proliferation index was calculated as percentage of Ki67-positive cells. Error bars represent SEM. A two-tailed unpaired t test was used for statistical analysis.

### **TUNEL staining**

For apoptosis detection, the ApopTag Red In Situ Apoptosis Detection Kit (S7165; Millipore) was used in accordance with the manufacturer's protocol.

### **Western Blotting**

Western blotting was performed as previously described (21). Detailed antibody information is included in Supplemental Methods, Table S1.

### **Quantitative RT-PCR**

Quantitative RT-PCR was performed as previously described (21). The primers are listed in Supplemental Methods, Table S2. Values were normalized to GAPDH as housekeeping gene expression control, and expressed as ratio over *E-cadherin/Cdh1* expression, to account for the different proportion of epithelium across different samples. A two-tailed unpaired t test was used for statistical analysis.

Detailed protocols and standard procedures are described in the Supplementary Methods.

## Results

### $\beta$ -catenin negative cells do not contribute to Kras driven PanIN lesions

To determine if  $\beta$ -catenin is required for PanIN formation, we generated Ptf1a-Cre; LSL-Kras<sup>G12D</sup>;  $\beta$ -catenin<sup>f/f</sup> (KBC) mice where both alleles of  $\beta$ -catenin are floxed in the context of mutant Kras (Supplemental Figure 1A). Recombination of the floxed  $\beta$ -catenin allele results in lack of functional protein (22). KBC mice survived at a slightly lower than Mendelian ratio, but then reached adulthood in apparent normal health. Pancreata dissected from 2-months-old KC mice had frequent PanIN lesions, surrounded by desmoplastic stroma (Supplemental Figure 1C). The pancreatic tissue in KBC mice presented with areas of fatty replacement surrounding clusters of normal acini and islets, acinar to ductal metaplasia (ADM) and PanINs (Supplemental Figure 1C).  $\beta$ -catenin is essential for acinar development (23) and thus the presence of fatty replacement likely reflects the loss of acinar cells during embryogenesis. In wild-type (WT) pancreata,  $\beta$ -catenin was localized at the cell membrane; accumulation of cytoplasmic and nuclear  $\beta$ -catenin was observed in PanIN lesions in KC mice, as previously reported (Supplemental Figure 1D)(9, 13). KBC tissues contained a mosaic of  $\beta$ -catenin positive and negative cells; of note mosaicism in the recombination of this floxed  $\beta$ -catenin allele has been described previously (23-25). Interestingly, ADM and PanIN lesions consisted uniquely of  $\beta$ -catenin positive cells;  $\beta$ -catenin negative cells persisted in the tissue as normal acinar or ductal cells (Supplemental Figure 1D). Occasionally,  $\beta$ -catenin negative cells were observed within dilated ducts. Invariably, they were small, had cuboidal morphology and lacked intracellular mucin, thus having characteristics of normal duct cells, even when surrounded by PanIN cells (Supplemental Figure 1B). Further analysis revealed that  $\beta$ -catenin knock-out clusters in KBC pancreata neither expressed CK19 and p-ERK1/2 nor displayed an increase in proliferation, unlike  $\beta$ -catenin positive clusters within the same KBC tissues and in control KC pancreata (Supplemental Figure 1E). Thus,  $\beta$ -catenin null cells did not appear to contribute to PanIN lesions.

Mutant Kras can promote PanIN formation by driving ADM, a process by which acinar cells dedifferentiate to a duct-like state capable of giving rise to PanIN lesions (10, 26). In order to determine if  $\beta$ -catenin negative acini were able to undergo ductal reprogramming, we used a 3-dimensional (3D) culture system. We isolated acinar clusters from adult, tamoxifen-treated Elastase-Cre<sup>ERT2</sup>;  $\beta$ -catenin<sup>f/+</sup> or Elastase-Cre<sup>ERT2</sup>;  $\beta$ -catenin<sup>f/f</sup> animals to study cells with specific deletion of  $\beta$ -catenin in the adult acinar compartment. The clusters were cultured in a collagen matrix and stimulated to undergo ADM with TGF- $\alpha$  treatment, as previously described (19). In control Elastase-Cre<sup>ERT2</sup>;  $\beta$ -catenin<sup>f/+</sup> ADM was widespread, with few remaining acinar clusters after 5 days of TGF- $\alpha$  treatment (Supplemental Figure 1F). In contrast, cultures derived from Elastase-Cre<sup>ERT2</sup>;  $\beta$ -catenin<sup>f/f</sup> mice displayed a mixture of ductal structures and persistent acinar clusters (Supplemental Figure 1F). By co-immunofluorescence, we observed that the ductal structures (expressing the ductal marker CK19) were nearly universally positive for  $\beta$ -catenin, while the remaining acinar clusters (identified by morphology and amylase expression) were predominantly formed by  $\beta$ -catenin null cells (Supplemental Figure 1G). Quantification of these results identified a striking change in cell fate between the  $\beta$ -catenin positive and negative cell population: cells expressing  $\beta$ -catenin were overwhelmingly found in the CK19+ (ductal) population, while virtually all the  $\beta$ -catenin null cells were CK19 negative (Supplemental Figure 1G). Therefore,  $\beta$ -catenin is required for acinar cells to undergo ADM. Taken together, these data provide, to our knowledge, the first functional evidence of the cell-autonomous requirement for intact  $\beta$ -catenin function during the onset of pancreatic carcinogenesis.

## Inhibition of ligand-dependent Wnt signaling inhibits PanIN formation

Since  $\beta$ -catenin is not only a key component of Wnt signaling, but also a structural component of the cadherin-based adherens junctions (27) we sought a different approach to block Wnt signaling during the onset of pancreatic carcinogenesis. Therefore, we utilized a different genetic approach to target canonical-Wnt signaling. We generated Ptf1a-Cre; Rosa26<sup>rtTa/+</sup>; TetO-Dkk1 mice (referred to as DKK1 mice, Supplemental Figure 2A), that allow inducible, doxycycline dependent expression of the secreted Wnt inhibitor Dkk1 in the pancreas. Dkk1 specifically inhibits canonical Wnt signaling by binding to the Kremen family of receptors, a process that results in endocytosis and thus elimination of the obligate canonical Wnt LRP5/6 co-receptors from the cell surface (28). First, we validated our system by administering doxycycline to 1-month-old DKK1 animals to induce Dkk1 expression (Supplemental Figure 2B). By activating Dkk1 in post-weaning mice, we were able to bypass the potential developmental effects of inhibiting Wnt signaling during pancreas development. Two months later, we observed widespread, but mosaic Dkk1 expression by immunohistochemistry (Supplemental Figure 2D). Histological analysis showed normal pancreatic histology, indicating that Dkk1 expression did not affect homeostasis of the adult pancreas (Supplemental Figure 2C).

To address the role of ligand-dependent Wnt signaling during PDA initiation, KC mice were crossed with Rosa26<sup>rtTa/rtTa</sup>(29) and TetO-Dkk1 (14) mice to generate KC; Rosa26<sup>rtTa/+</sup>; TetO-Dkk1 quadruple transgenics, hereby referred to as KDC (Figure 1A). In absence of doxycycline the disease progression in KDC mice was histologically indistinguishable from that of KC mice (Supplemental Figure 2E, 2F). Then, we expressed Dkk1 in the adult pancreatic epithelium of KDC mice, starting at 1 month of age (Figure 1B) when KC and KDC mice only have rare PanINs (7, 30); thus, in this experiment, Dkk1 expression preceded PanIN formation. Doxycycline water was continuously administered to KC and KDC littermates until the pancreatic tissue was harvested. Based on the expected disease progression in KC mice, we harvested tissue after 1, 2, 5 and 8 months (Figure 1B) with a minimum of 4 animals per genotype per time point. One month following doxycycline administration, the pancreatic parenchyma in KC mice was almost completely replaced by PanIN lesions, ranging from PanIN1A to PanIN2, with occasional PanIN3 lesions. In contrast, in age-matched KDC mice, most of the pancreas was composed of normal acini, with rare PanINs, mainly PanIN1A (Figure 1C). Both KC and KDC mice showed areas of ADM. The analysis of tissues two months after doxycycline administration showed more, and higher-grade PanINs in KC animals, but still mostly normal acini and ADM in KDC littermates. At later time points (5 and 8 months after doxycycline administration), however, PanINs ranging from PanIN1A to PanIN2, interspersed between normal acini and ADM were also observed in KDC mice (Figure 1C). At these stages KC littermates had a higher percentage of PanIN2 and PanIN3 with almost no normal acini and much fewer ADM lesions. Histopathological quantification confirmed that KDC mice had fewer PanINs overall, and lower grade lesions than KC littermates at all of the time points (Figure 1D). Summarily, KDC mice displayed inhibited PanIN development and progression.

The inhibitory effect on PanIN formation observed in KDC mice, however, diminished over time (Figure 1C), as revealed by the progressive increase in several PanIN markers (Supplemental Figure 3A-C and Supplemental Figure 4A, 4B). Of note, similarly low levels of apoptosis was observed in PanIN lesions in KC and KDC mice (Supplemental Figure 4C).

We then determined the ability of Dkk1 to inhibit Wnt/ $\beta$ -catenin signaling in the pancreas. Expression of Dkk1 was confirmed in KDC, but not KC mice by western blot (Figure 2B). We then measured the levels of total  $\beta$ -catenin, which accumulates in presence of active Wnt signaling, and phospho- $\beta$ -catenin, present when the Wnt pathway is not active. In KC

animals, PanINs showed high levels of total  $\beta$ -catenin and very little to no phospho- $\beta$ -catenin; in contrast, KDC tissues had lower levels of total  $\beta$ -catenin but increased levels of phospho- $\beta$ -catenin at all the time points (Figure 2B and 2C). However, at 8 months, expression of *Dkk1* was lower in KDC mice, and correspondingly we observed less phospho- $\beta$ -catenin. Finally, we analyzed the expression of Wnt target genes and pathway components in KC and KDC mice, comparing cohorts of 3 month-old animals that had been on doxycycline for 2 months. As expected, *Dkk1* expression was higher in KDC pancreata; the Wnt target genes *Axin2*, *Lef1*, *MMP7*, and the Wnt pathway components *Wnt2*, *Fzd1* and *Fzd2* had lower expression in KDC tissues, consistent with reduced Wnt signaling. The effect was not due to overall reduced transcription as we observed no change in other Wnt pathway components not regulated by canonical pathway activity, such as *LRP5*, *LRP6* and *Wnt3a*, among others (Figure 2D and data not shown). Additionally, the Hedgehog pathway ligands Sonic Hedgehog (*Shh*) and Indian Hedgehog (*Ihh*) and the Notch target gene *Hes1* were also significantly reduced in KDC tissues compared to KC (Figure 2D), consistent with the lower PanIN load in these animals. Thus, our data show that *Dkk1* expression efficiently blocks activation of the Wnt signaling pathway and inhibits PanIN formation, therefore providing evidence that activation of the Wnt/ $\beta$ -catenin pathway is required to initiate pancreatic carcinogenesis. To determine whether the inhibition of the Wnt pathway was maintained over time, we analyzed the expression of the Wnt target gene *Axin2* (Figure 2D). Initially *Axin2* expression was lower in KDC than in KC animals, but its expression later rebounded in KDC mice. In fact, *Axin2* expression was significantly higher in KDC than KC mice at the 8 month on doxy time point, a finding that might reflect the different cellular composition of the tissues in each cohort at this time point and possibly activation of the pathway in non-epithelial cell types, or positive selection for cells expressing higher levels of Wnt components.

To determine whether inhibition of Wnt signaling prolonged overall survival, we aged a cohort of KC (n=46) and KDC (n=24) mice on doxycycline from one month of age. While only about 1/3 of the KC mice developed frank adenocarcinoma, most animals were sacrificed due to declining body condition within one year of age and presented at autopsy with PanIN3 and severe exocrine insufficiency due to loss of the pancreas parenchyma. KDC mice had significantly longer survival (Supplemental Figure 5A). We observed invasive tumors with liver metastases both in KC and KDC mice and lung metastases only in the KC cohort. Tumors from KC and KDC mice were highly proliferative, expressed phospho-ERK1/2, *Muc1* and *MMP7*, and displayed accumulation of desmoplastic stroma (Supplemental Figure 5B, 5C). We also observed accumulation of  $\beta$ -catenin indicating active Wnt pathway. Furthermore, immunostaining for *Dkk1* in the KDC tumor revealed that its expression was lost, in contrast with the surrounding tissue (Supplemental Figure 5C). These findings suggest that the invasive tumor either originated from cells that had never recombined the *rtTa* transgene, thus never expressing *Dkk1*, or from cells with inactivated *Dkk1* expression possibly due to transgene silencing or insufficient doxycycline exposure.

### **Treatment with the OMP-18R5 therapeutic monoclonal antibody recapitulates *Dkk1* overexpression**

Given the genetic evidence of the ability of Wnt inhibition to prevent PanIN formation, as shown above, we sought to complement our studies by using a therapeutically relevant approach. Namely, we used the monoclonal antibody OMP-18R5 that binds to multiple Frizzled receptors and blocks their activity (16). 1-month old KC mice were treated with OMP-18R5 (10mg/Kg, twice/week) or isotype control by intraperitoneal injection for 2 months, and then sacrificed (Figure 3A). As a biomarker of the OMP-18R5 activity, we used hair growth, as Wnt/ $\beta$ -catenin signaling in hair follicle precursor cells serves as a crucial

signal for the telogen-anagen transition in mice (31). Treatment with OMP-18R5 prevented hair growth in KC mice after depilation, indicating effective Wnt inhibition (Supplemental Figure 6B). Two months after initiating the study, histological analysis of the pancreas revealed extensive PanINs, including high-grade lesions, in control KC animals, while KC mice treated with OMP-18R5 had a prevalence of normal acini and ADM, and fewer lesions (Figure 3B and quantification in Figure 3C). Importantly, we observed an increase in phospho- $\beta$ -catenin, indicating effective inhibition of Wnt signaling (Figure 3B). In dissected tissues, pathway inhibition was confirmed by RT-qPCR for the Wnt target genes Axin2, Lef1 and MMP7 (Figure 3D). Moreover, we detected lower phospho-ERK1/2 in OMP-18R5-treated samples, and a significant decrease in ki67 positive cells (Figure 3B). Thus, treatment with OMP-18R5 recapitulated the effect of Dkk1 overexpression, and validated the role of ligand-driven Wnt signaling in PanIN formation.

OMP-18R5 binds multiple Frizzled receptors and thus potentially inhibits both canonical and non-canonical Wnt signaling. In addition, inhibition of canonical Wnt signaling might lead to redirection of the signaling towards the non-canonical pathway through a feedback mechanism (32). Therefore we examined if OMP-18R5 treatment also affected non-canonical Wnt signaling. We measured the expression of the core component of the planar cell polarity (PCP) pathway, Vangl2 (33) and the core component of Wnt/Ca<sup>2+</sup> pathway Cdc42 (34), as well as the non-canonical ligand Wnt5a and found no significant change in KDC mice or KC mice treated with OMP18R5 compared to control (Figure 3E).

### Cross-talk of Wnt/ $\beta$ -catenin and MAPK/ERK signaling during PanIN formation

Activation of the MAPK/ERK pathway accompanies, and is necessary for, PanIN formation (35, 36); even in presence of oncogenic Kras, activation of the MAPK/ERK pathway requires additional stimuli, such as inflammation or EGF signaling (4, 37-42). We measured phospho-ERK1/2 as readout of the MAPK/ERK pathway activation in KC and KDC mice at the time points indicated in Figure 4A. Phospho-ERK1/2 was highly expressed in PanINs in KC pancreata (Figure 4B, 4C). In contrast, KDC pancreata had lower levels of phospho-ERK1/2 as measured by immunohistochemistry and Western blot. Intriguingly, even the levels of total ERK1/2 protein were slightly lower in KDC pancreata than in KC animals, possibly indicating additional levels of regulation besides phosphorylation. However, when PanIN lesions were observed in KDC tissues, they had high expression of phospho-ERK1/2, comparable to that observed in KC tissues (Figure 4C, see 5 months and 8 months time points). To further investigate this finding we performed co-immunofluorescence for CK19 (a ductal marker expressed in ADM and PanINs), Dkk1, and phospho-ERK1/2. In 2 month-old KDC animals cells expressing Dkk1 were mostly CK19 negative (Figure 4D). In 9 month-old KC mice we frequently observed higher-grade PanIN lesions with high phospho-ERK1/2 expression. High-grade PanIN lesions were rare in age matched KDC animals, and those lesions that were present were largely negative for Dkk1 expression (Figure 4D). In 9 month-old KDC mice, Dkk1 was expressed in ADM and low-grade PanINs, but like the 2-month time point, cells expressing Dkk1 were largely CK19 negative, and cells co-expressing Dkk1 and CK19 generally displayed low or near undetectable levels of phospho-ERK1/2 expression. Taken together, our data indicates that Dkk1 expressing cells inefficiently undergo Kras driven ADM; moreover, even if they undergo ADM they rarely up-regulate phospho-ERK1/2 correlating with impaired PanIN specification and progression to higher lesion grade. Thus, the increase in Wnt target gene expression and phospho-ERK1/2 positive lesions in older KDC mice might be explained by a progressive loss of Dkk1-expressing cells – possibly due to proliferation disadvantage.

We then investigated the Wnt/MAPK crosstalk in established pancreatic cancer cells. Primary mouse pancreatic cancer cells (65671) were derived from tumor-bearing KPC mice (Ptf1a-Cre; LSL-Kras<sup>G12D</sup>; p53<sup>flox/+</sup>) inbred in the FVBN1 strain; they are a pure epithelial

and highly tumorigenic population. 65671 cells expressed Wnt ligands and had active Wnt signaling. Treatment of 65671 cells in culture with recombinant rDkk1 or OMP-18R5 led to inhibition of Wnt activity, as determined by decrease in expression of the Wnt target gene Axin2 (Figure 4E). Interestingly, rDkk1 or OMP-18R5 treatment also led to down-regulation of the MAPK signaling pathway (as indicated by reduced phospho-ERK1/2 levels), but had no effect on phospho-Akt (Figure 4F). We then investigated non-canonical Wnt signaling in response to OMP-18R5 treatment in this panel of primary tumor cells by measuring the level of activation of two kinases, JNK and PKC, which mediate the Wnt/PCP and Wnt/Ca<sup>2+</sup> signaling branches respectively (for review see (43)). We observed no changes in p-JNK levels, and correspondingly no change in the expression level of Vangl2 (Supplemental Figure 6C, 6E). In contrast, p-PKC levels transiently decreased upon treatment with OMP-18R5 (Supplemental Figure 6C), a finding that might indicate changes in the Wnt/Ca<sup>2+</sup> signaling pathway.

In order to determine whether the cross-talk between the Wnt and MAPK pathway exists in human tumors, we used the primary human PDA cell lines UM2, 1319, UM18 and UM19 (18). Wnt inhibition resulted in down-regulation of p-ERK1/2 and p-Akt (Supplemental Figure 6C). Thus, even in primary human tumor cells, Wnt signaling is a positive regulator of the MAPK pathway. Using RT-PCR of canonical Wnt-targets to verify pathway inhibition, treatment with rhDkk1 only inhibited Axin2 expression in two of the 4 cell lines tested and OMP-18R5 was effective in three out of the 4 cell lines (Supplemental Figure 6F). Similarly, among three primary mouse pancreatic cancer cell lines, both agents inhibited Axin2 expression in two lines, 65671 and 4292, while only OMP-18R5 inhibited Axin2 expression in the third line 9805 (Supplemental Figure 6D). The different response of individual cell lines to Dkk1 and OMP-18R5 might reflect biological differences such as mutations in signaling components that affect the functionality of each inhibitor differently, based on their different mechanisms of action.

### **Wnt/ $\beta$ -catenin signaling regulates pancreatic cell proliferation and Kras driven acinar cell reprogramming**

The adult pancreas has a very low frequency of Ki67 positive cells, as most cells are not dividing (Supplemental Figure 7C). Proliferation was similarly low in Dkk1 expressing pancreata (Supplemental Figure 7C). In KC animals, significantly increased proliferation – both in the epithelial compartment and in the surrounding stroma – accompanies PanIN formation (Supplemental Figure 7B, 7C). In contrast, we observed a decrease in proliferation in KDC tissues, specifically in the epithelial compartment (Supplemental Figure 7B, 7C), both in ductal structures (ADM and PanINs) and acinar cells (Supplemental Figure 7D). Therefore, our data suggests that active Wnt signaling supports Kras dependent epithelial proliferation during the early steps of pancreatic carcinogenesis.

We then investigated the effect of Wnt signaling inhibition on the onset of ADM. To follow a specific cell cluster over time, we used 3D cultures of acinar clusters in Matrigel. Wild-type and KC acinar clusters spontaneously underwent ADM in Matrigel; however, the formation of duct-like structures was significantly faster and more penetrant in KC acini (Figure 5A, 5B). Moreover, KC-derived ductal structures accumulated more intraluminal debris and more intracellular mucin over time (as shown by PAS staining, Figure 5C). Formation of ductal structures in KDC-derived cultures was equivalent to wild-type, with slower and less penetrant duct formation compared to KC clusters (Figure 5A quantification in Figure 5B). Moreover, mucin accumulation – an indication of progression from ADM to PanIN – was reduced in KDC, compared to KC clusters (Figure 5C). Thus, Dkk1 expression inhibited the capacity of Kras to drive acinar to ductal reprogramming and enforce an aberrant mucinous program in metaplastic ducts. This set of data was consistent with our in

vivo observation that both Kras driven ADM formation and progression to PanINs are inhibited in KDC pancreata.

### Inhibition of Wnt signaling in established PanINs

The inducible Dkk1 expression in KDC mice gave us the opportunity to investigate the effect of Wnt inhibition in established PanINs. In this set of experiments, doxycycline was administered to 2 month-old KC and KDC animals (n=4), when a discrete number of PanINs lesions can be observed in the tissues (Supplemental Figure 2E, 2F). The animals were euthanized 6 weeks later and the pancreas was dissected (see scheme in Figure 5D), and pancreas size was measured as a proxy for disease progression as previously described (44). KDC mice had a 2-fold smaller pancreas compared to KC littermates, reflecting reduced pancreas growth over time (Figure 5E, 5G). Histological analysis of the tissue did not reveal any significant morphological difference between the two cohorts (Figure 5G, H&E staining); however, Ki67 staining indicated a greater than 2-fold, in the proliferation index of KDC tissues compared to KC (Figure 5G, and quantification in Figure 5F) both in the epithelial compartment and in the surrounding stroma. Moreover, the number of apoptotic cells in the stroma of KDC animals (measured by cleaved caspase 3 and by TUNEL) was higher in KDC tissues (Supplemental Figure 8), suggesting that Wnt signaling might exert a paracrine, pro-survival effect on the tumor stroma. Therefore, Wnt signaling inactivation blocks proliferation of established PanIN lesions and may contribute to maintenance of desmoplasia during PDA development.

### Discussion

Wnt signaling regulates numerous aspects of pancreatic biology. During pancreas development, both inappropriate activation and inactivation of the pathway lead to profound defects of organ formation, including pancreatic agenesis (23, 45, 46). Moreover,  $\beta$ -catenin, the critical transcriptional node in canonical Wnt signaling, is essential for exocrine regeneration following damage such as induction of acute pancreatitis in mice (10, 47). Wnt signaling activity is gradually increased during pancreatic carcinogenesis (9, 10), but the levels of activation are within physiological limits even in advanced tumors. In fact, it was previously shown that forced high-level activation of the pathway prevents Kras driven specification of acinar derived PanINs (10) and diverts pancreatic cells undergoing Kras driven transformation from the PanIN/PDA progression towards an unusual tumor type, reminiscent of rare human intraductal tubular tumors (ITT) (48). Pancreatic cancer cells express several Wnt ligands (9, 49, 50), possibly indicating autocrine activation of the pathway. Moreover, mutations in Wnt signaling components have been identified in the majority of human pancreatic tumors (3), as well as in a mouse screen for mutations synergizing with Kras in driving pancreatic carcinogenesis (51). The exact biological significance of each of the mutations identified is in need of further exploration. In addition to canonical Wnt signaling, non-canonical signaling may also play an important role in this disease (52-56). The relative importance the different modalities of Wnt signaling requires further investigation.

Here, we show that inhibition of Wnt signaling significantly delayed PanIN formation. Taken together, our results suggest that there is a minimal threshold of ligand mediated, canonical Wnt signaling required for PanIN formation (Figure 6). In contrast, high levels of Wnt activation prevent specification of the PanIN-PDA lineage (10, 48) (Figure 6). In the future, genetic models that permit controllable, graded increases in Beta-catenin activity, or inducible expression of Wnt ligands will elucidate the threshold at which canonical Wnt signaling is no longer compatible with Kras driven PanIN development.

An essential step for the initiation of pancreatic carcinogenesis is activation of the MAPK/ERK signaling pathway. In KC mice, activation of MAPK signaling is a key step for PanIN formation (35). Mutant Kras and Wnt signaling pathway cooperate to drive tumorigenesis in other tissues, for example in lung and colon adenocarcinoma (57, 58). Epistatic interactions between MAPK and Wnt signaling have been recently described both in melanoma and in colon cancer (59, 60). Intriguingly, these interactions are clearly tissue-specific: in melanoma Wnt signaling inhibits MAPK signaling (60) while in colon cancer Wnt signaling stabilizes Ras and thus promotes MAPK activity (59). Here, we show that Wnt signaling in the pancreas promotes MAPK signaling and has a tumor-promoting effect. Thus, the identity of  $\beta$ -catenin targets that antagonize or promote Kras dependent transformation may be tissue specific, and thus may provide unique cancer specific therapeutic targets.

Studying the role of Wnt/ $\beta$ -catenin during the initiation of PDA highlighted the potential role of targeting Wnt/ $\beta$ -catenin signaling in disease initiation. This finding has implications for the potential use of Wnt inhibitors in pancreatic cancer patients, at a time when several Wnt inhibitors, including OMP-18R5 have entered the clinical pipeline for treatment of a variety of cancers, including PDA [for review, see (61, 62)]. OMP-18R5 has been found to be active in combination with a variety of chemotherapeutic agents in patient-derived xenografts, including gemcitabine in PDA xenografts (16). Our data indicate that the mechanism of action might involve a reduction in the activation of the MAPK/ERK signaling, and inhibition of proliferation. Further studies might elucidate whether interactions between the tumor cells and the surrounding stroma are also regulated through Wnt signaling.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

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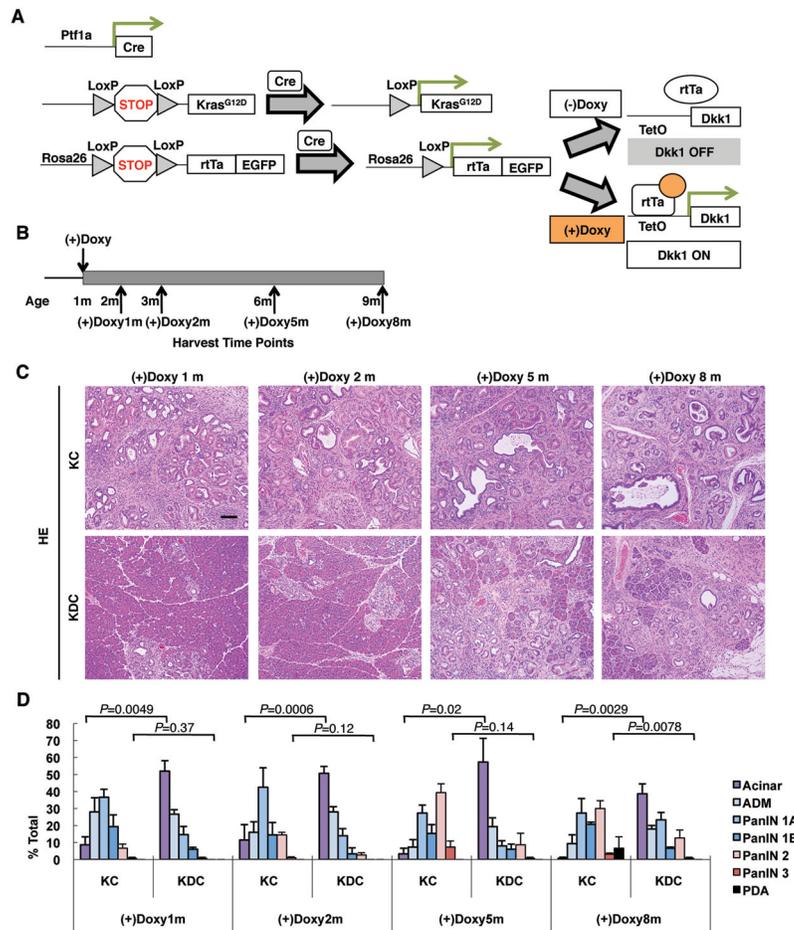
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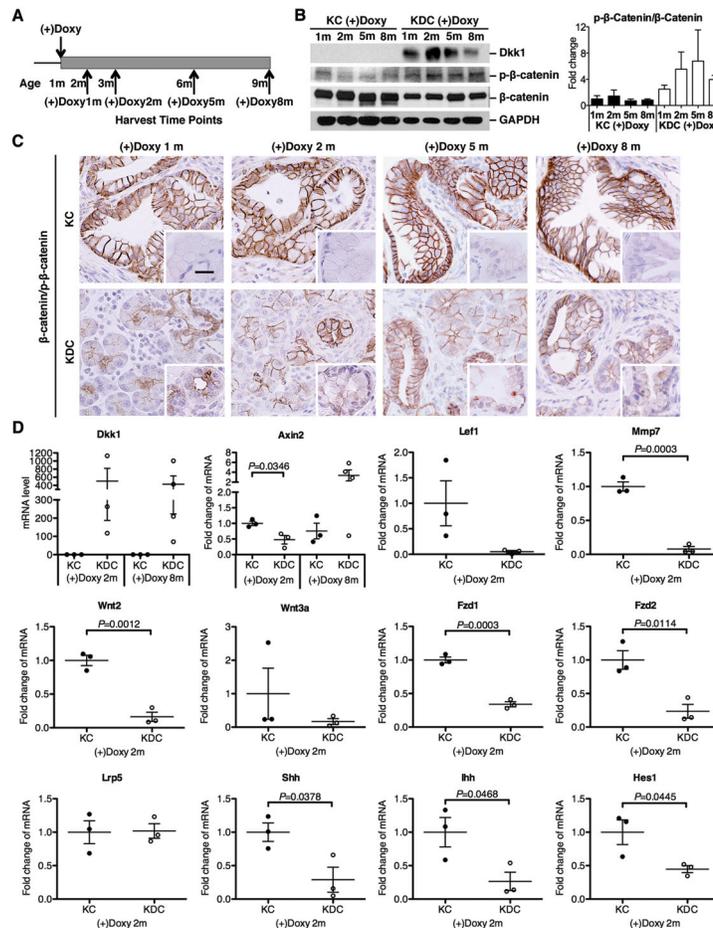
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**Figure 1. Inhibition of the Wnt/ $\beta$ -catenin signaling by Dkk1 prevents PanIN formation.**

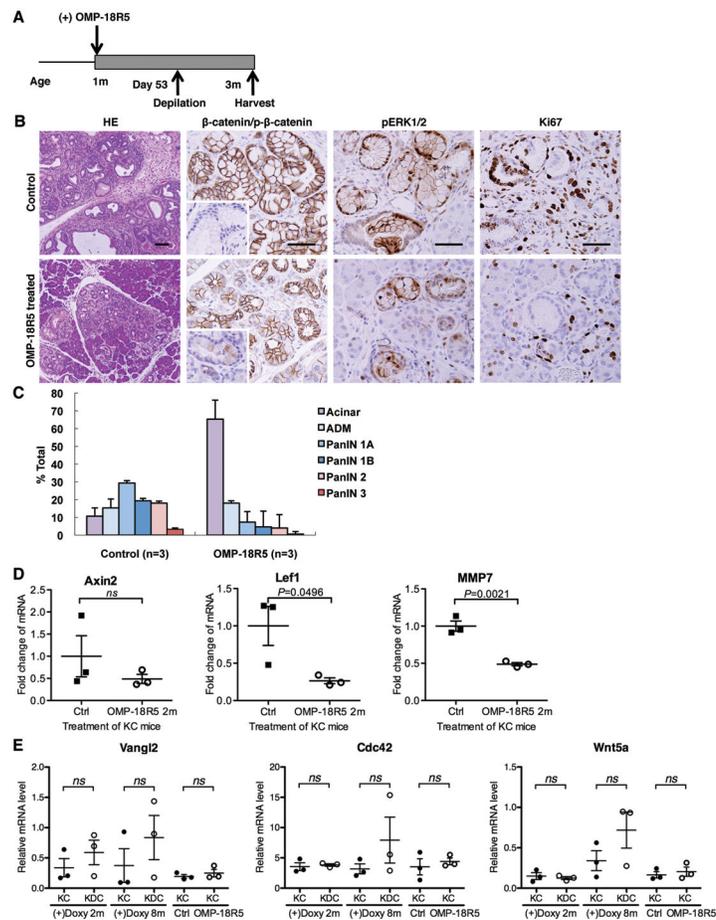
**Figure 1. Inhibition of the Wnt/ $\beta$ -catenin signaling by Dkk1 prevents PanIN formation**  
 (A) Genetic makeup of the KDC model. (B) Experimental design. (C) H&E staining of KC and KDC pancreata at 1 month, 2 months, 5 months and 8 months following induction of Dkk1 expression. Scale bar 100 $\mu$ m. (D) Pathological analysis. Data represent mean  $\pm$  SEM, n=4. The statistical difference was determined by two-sided Student's t-test.



**Figure 2. Dkk1 expression inhibits Wnt/ $\beta$ -catenin signaling.**

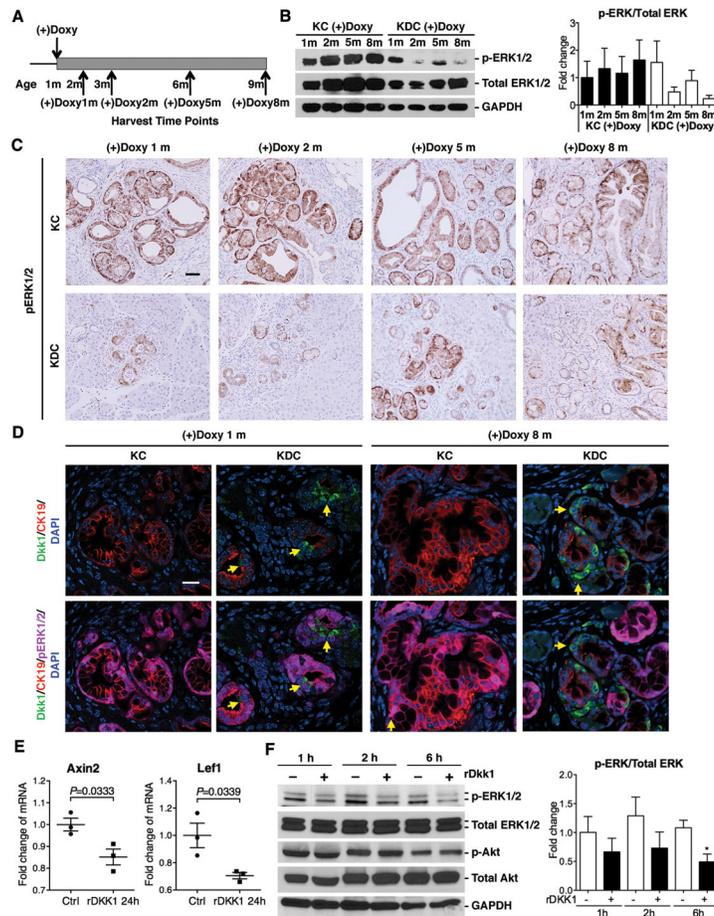
**Figure 2. Dkk1 expression inhibits Wnt/ $\beta$ -catenin signaling**

(A) Experimental design. (B) Western blot for Dkk1,  $\beta$ -catenin and phospho- $\beta$ -catenin in KC and KDC pancreas; quantification of the fold change of phospho- $\beta$ -catenin/ $\beta$ -catenin. Data represent mean  $\pm$  SEM, n=3. (C) Immunohistochemistry for  $\beta$ -catenin and phospho- $\beta$ -catenin (insert). Scale bar 20  $\mu$ m. (D) RT-qPCR analysis of Dkk1, Wnt/ $\beta$ -catenin target genes and component. Each dot represents one mouse. Data represent mean  $\pm$  SEM. The statistical difference was determined by two-sided Student's t- test.



**Figure 3. Inhibition of the Wnt/β-catenin signaling by OMP-18R5 prevents PanIN formation.**

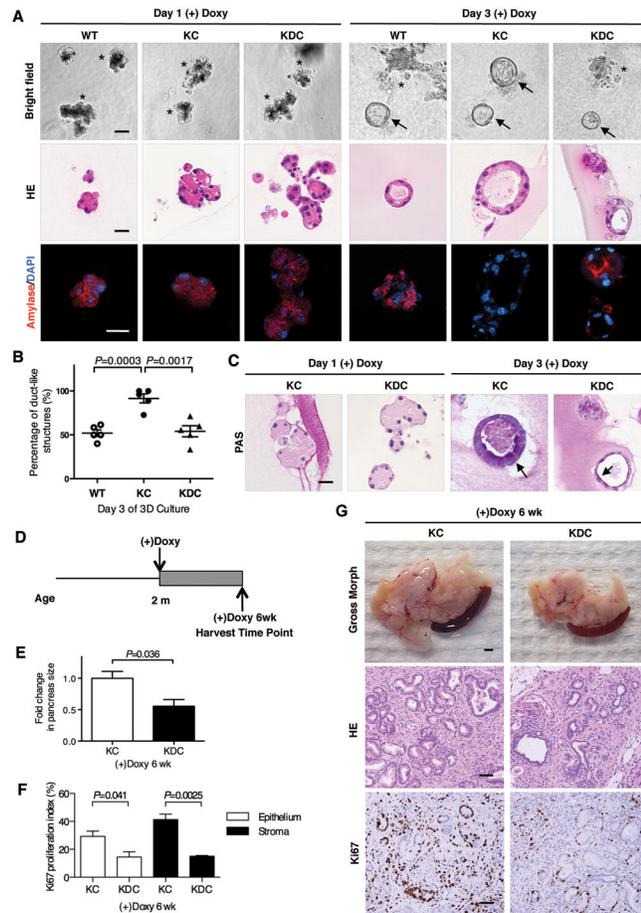
**Figure 3. Inhibition of the Wnt/β-catenin signaling by OMP-18R5 prevents PanIN formation**  
 (A) Experimental design for OMP-18R5 treatment; n=5 mice per cohort. (B) H&E staining and immunohistochemical analysis for β-catenin, phospho-β-catenin, phospho-ERK1/2 and ki67. Scale bar 50μm. (C) Quantification of the lesions. (D) RT-qPCR analysis of Wnt/β-catenin target genes. (E) RT-qPCR analysis of non-canonical Wnt signaling components. Each dot represents one mouse. Data represent mean ± SEM. The statistical difference was determined by two-sided Student's t- test.



**Figure 4. Cross-talk of Wnt and MAPK signaling pathways.**

**Figure 4. Cross-talk of Wnt and MAPK signaling pathways**

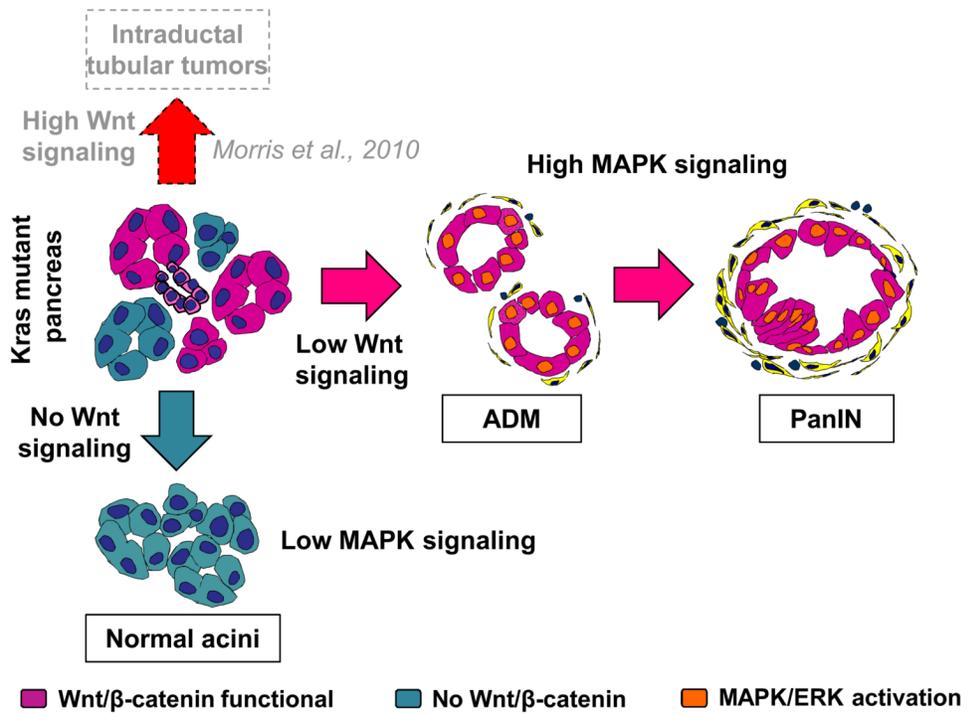
(A) Experimental design; n=3 to 5 mice per time point. (B) Western blot for ERK1/2 and phospho-ERK1/2; quantification of the fold change of phospho-ERK/Total ERK. Data represent mean  $\pm$  SEM, n=3. (C) Immunohistochemistry for phospho-ERK1/2. Scale bar 50 $\mu$ m. (D) Co-immunofluorescence for Dkk1 (green), CK19 (red), pERK1/2 (magenta) and DAPI (blue). Arrows: Dkk1 positive cells. Scale bar 25 $\mu$ m. Primary pancreatic cancer cells 65671 were treated with rDkk1: (E) RT-qPCR for Wnt/ $\beta$ -catenin target genes Axin2 and Lef1. Data represent mean  $\pm$  SEM, n=3. The statistical difference was determined by two-sided Student's t- test. (F) Western blot for phospho-ERK1/2, total ERK, phospho-Akt, total Akt and GAPDH.



**Figure 5. Wnt signaling is required for Kras-driven acinar-ductal metaplasia and PanIN proliferation.**

### Figure 5. Wnt signaling is required for Kras-driven acinar-ductal metaplasia and PanIN proliferation

(A) Transmitted light images, H&E staining and Amylase immunofluorescent staining of KC and KDC pancreatic cell clusters in 3D culture. Arrows: duct structures, asterisks: acinar clusters. Scale bar 20 $\mu$ m. (B) Quantification of duct-like structures at day 3. Data represent mean  $\pm$  SEM, n=5. The statistical difference was determined by two-sided Student's t- test. (C) PAS staining. Arrows: PAS positive cells. Scale bar 20 $\mu$ m. (D) Experimental design; n=4 mice. (E) Pancreas size (mean  $\pm$  SEM, n=4). The statistical difference was determined by two-sided Student's t-test. (F) Proliferation index in epithelial and stromal compartments. Data represent mean  $\pm$  SEM, n=4. The statistical difference was determined by two-sided Student's t-test. (G) Gross morphology of KC and KDC pancreata (scale bar 2mm); H&E and Ki67 staining (scale bar 50 $\mu$ m).



**Figure 6. Wnt/β-catenin signaling in Kras-driven pancreatic carcinogenesis**

A physiological level of Wnt signaling (low Wnt signaling, magenta arrow) is maintained during the formation of pancreatic cancer precursor lesions. If Wnt signaling levels are altered, either by overexpression (red arrow) or by inactivation (blue arrow), pancreatic carcinogenesis is prevented.