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PI3K Regulation of RAC1 is Required for KRAS-induced Pancreatic Tumorigenesis in Mice

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Abstract

Background & Aims—New drug targets are urgently needed for the treatment of pancreatic ductal adenocarcinoma (PDA). Nearly all PDAs contain oncogenic mutations in the *KRAS* gene. Pharmacologic inhibition of KRAS has been unsuccessful, leading to a focus on downstream effectors that are more easily targeted with small molecule inhibitors. We investigated the contributions of phosphoinositide 3 kinase (PI3K) to KRAS-initiated tumorigenesis.

Methods—Tumorigenesis was measured in the *Kras*^{G12D/+};*Ptfla*^{Cre/+} mouse model of PDA; these mice were crossed with mice with pancreas-specific disruption of genes encoding PI3K p110α (*Pik3ca*), p110β (*Pik3cb*), or RAC1 (*Rac1*). Pancreatitis was induced by 5 daily intraperitoneal injections of cerulein. Pancreata and primary acinar cells were isolated; acinar cells were incubated with an inhibitor of p110α (PIK75) followed by a broad spectrum PI3K inhibitor (GDC0941). PDA cell lines (NB490 and MiaPaCa2) were incubated PIK75 followed by GDC0941. Tissues and cells were analyzed by histology, immunohistochemistry, quantitative reverse transcription PCR, and immunofluorescence analyses for factors involved in the PI3K signaling pathway. We also examined human pancreas tissue microarrays for levels of p110α and other PI3K pathway components.

Results—Pancreas-specific disruption of *Pik3ca* or *Rac1*, but not *Pik3cb*, prevented development of pancreatic tumors in *Kras*^{G12D/+};*Ptfla*^{Cre/+} mice. Loss of transformation was independent of AKT regulation. Preneoplastic ductal metaplasia developed in the mice lacking pancreatic p110α, but regressed. Levels of activated and total RAC1 were higher in pancreatic tissues from *Kras*^{G12D/+};*Ptfla*^{Cre/+} mice, compared with controls. Loss of p110α reduced RAC1 activity and expression in these tissues. p110α was required for the upregulation and activity of the RAC guanine exchange factor (encoded by *Rasgrf1*) during tumorigenesis. Levels of p110α and RAC1 were increased in human pancreatic intraepithelial neoplasias and PDAs, compared with healthy pancreata.

Conclusions—KRAS signaling, via p110α to activate RAC1, is required for transformation in *Kras*^{G12D/+};*Ptfla*^{Cre/+} mice.

Keywords

pancreatic cancer; signal transduction; cytoskeleton; oncogene

INTRODUCTION

Pancreatic ductal adenocarcinoma (PDA) is the fourth leading cause of cancer-related death worldwide, with a 6% five year survival rate¹. Chemotherapy and radiation therapy have little impact on survival, leaving surgery as the most effective treatment². Unfortunately, only 15% of patients who undergo surgery survive past five years^{3,4}. This bleak outcome makes understanding the molecular mechanisms of PDA formation and progression so that new “druggable” targets can be identified of paramount importance.

Nearly all PDAs harbor oncogenic mutations in the *KRAS* gene⁵, and pancreatic tumor maintenance is compromised when KRAS signaling is extinguished^{6,7}. While pharmacological inhibition of KRAS has thus far been unsuccessful⁸, oncogenic KRAS

activates downstream kinase effectors such as RAF-MEK and PI3K-AKT that are more easily targeted with small molecule inhibitors. Inhibition of one downstream target, MEK, blocks formation of tumor precursors known as metaplastic ducts, thus limiting subsequent transformation^{9–11} and can regress mPanINs¹¹. In this study, we focused on phosphoinositide 3-kinase (PI3K)^{12, 13} and its role in Kras-induced pancreatic tumorigenesis.

Class I PI3Ks are heterodimers consisting of a p110 catalytic subunit (α , β , δ or γ) bound to a regulatory subunit. Upon activation, they phosphorylate phosphatidylinositol 4,5-bisphosphate (PIP₂) to generate the lipid second messenger phosphatidylinositol-3,4,5-trisphosphate (PIP₃)¹⁴. PIP₃ binds to adaptor and effector proteins containing pleckstrin homology (PH) domains, driving several downstream signaling cascades. Two well-understood PI3K downstream effectors are the protein kinase AKT¹⁵ and the small G-protein RAC¹⁶. AKT and phosphoinositide-dependent kinase 1 (PDK1) are relocalized to PIP₃-containing membranes where PDK1 phosphorylates and activates AKT¹⁷. PI3K primarily activates RAC by relocalization of PH-domain containing guanine nucleotide exchange factors (GEFs) to the plasma membrane¹⁶.

While it is accepted that Ras proteins activate PI3K, the precise role of PI3K signaling in Kras-induced PDA is controversial. Eser et al., demonstrate that transgenic expression of a constitutively active mutant of p110 α (H1047R) is sufficient to induce pancreatic tumorigenesis¹⁸, contrary to results reported in a similar mouse model¹⁹. PDK1 is essential for Kras-induced PDA, attributed to a dependency of requisite acinar-to-ductal metaplasia (ADM) prior to tumorigenesis¹⁸. RAC1 is also required for pancreatic tumorigenesis by regulating morphological changes that accompany ADM²⁰.

Of the catalytic isoforms, p110 γ is expressed primarily by hematopoietic cells²¹, but is expressed in pancreatic acinar cells, where it controls cellular responses in experimental pancreatitis²². p110 δ is considered hematopoietic cell specific²¹. p110 α and p110 β are ubiquitously expressed²¹, and have been shown to be critical for tumorigenesis in other glandular tissues, such as breast²³, lung²⁴ and prostate²⁵. Here, we test whether p110 α or p110 β are involved in Kras-induced pancreatic tumorigenesis using isoform specific knockout mice. We demonstrate that the p110 α catalytic subunit is required for pancreatic tumorigenesis in a PDA mouse model, whereas the p110 β subunit is unnecessary. Remarkably, p110 α was not required for AKT activation due to compensation by other p110 catalytic subunits. However, p110 α -null pancreata were incapable of forming stable metaplastic ducts *in vivo*, which phenocopies *Rac1*-null mice. Consistent with this, we found that p110 α was required for robust RAC1 activation, in part by regulating acinar cell RAC GEF activity.

MATERIALS AND METHODS

Animal Models

Kras^{LSL-G12D/+}, *Ptf1a*^{Cre/+}, *Trp53*^{+R172H}, *p110 α* ^{Flox/Flox}, *p110 β* ^{Flox/Flox} and *Rac1*^{Flox/Flox} strains have been described^{26–29}. Experiments were conducted in accordance with the Office

of Laboratory Animal Welfare and approved by the Institutional Animal Care and Use Committees of Stony Brook University and the Mayo Clinic.

Antibodies

A complete list of antibodies used is provided in the Supplemental Information.

Cerulein Treatment

250µg/kg cerulein (American Peptide Company) were administered daily, intraperitoneally (i.p.) for 5 days, with 24 hours or 7 days recovery. To assess cytoskeletal rearrangement, 30 day old mice were injected i.p. with 250µg/kg cerulein for 3 days and sacrificed 1 hour following the last injection.

Immunofluorescence Staining

Immunofluorescence was performed on frozen sections of PFA perfused pancreata as described previously³⁰. Images were acquired on a Zeiss 510LS Meta confocal microscope (Carl Zeiss). Colocalization was determined by Zen 2012 v8.0 software (Carl Zeiss).

Histology and IHC

Pancreata were fixed overnight in 4% PFA. Tissues were processed in a Leica ASP300S Tissue Processor, paraffin-embedded and cut into 5 µm sections. IHC for phospho-Akt (T308), p110α, Vav1, Tiam1 and CK19 was performed on a Discovery Ultra XT autostainer (Ventana Medical Systems) and counterstained with hematoxylin. Dual IHC for CK19 and Amylase was performed without counterstain. IHC staining for RAC1 was performed on a DAKO Autostainer Plus (DAKO North America, Inc.) and counterstained with hematoxylin. Picrosirius red staining was performed per manufacturer's instructions (Polysciences, Inc.). Hemotoxylin and Eosin staining was performed using Mayer's Hemotoxylin solution (Sigma) and Eosin Y (Fisher).

Immunoblotting

For lysate preparation, the pancreas was removed from 12–14-week old mice and immediately homogenized in MLB buffer (25mM HEPES, pH 7.5, 150mM NaCl, 10mM MgCl₂, 1mM EDTA, 1% NP-40, and 10% glycerol) containing protease and phosphatase inhibitors (Roche) using a Pro 250 Homogenizer (Pro Scientific). For pancreas lysate immunoblots, 50 µg of protein was separated by denaturing SDS-PAGE, transferred to Immobilon P (Millipore) and visualized by chemiluminescence. Isolated acinar cells were washed once in PBS then lysed in MLB buffer containing protease inhibitors. 75 µg of protein was separated by SDS-PAGE, transferred to Immobilon P (Millipore) and visualized by chemiluminescence. Cell lines were washed once in PBS then lysed in RIPA buffer (150mM NaCl, 10mM Tris pH 7.2, 1% sodium deoxycholate, 1% Triton X-100, and 0.1% SDS) containing protease and phosphatase inhibitors. 25 µg of protein was separated by SDS-PAGE and visualized by chemiluminescence.

Active Ras, Rac and Rac-GEF Assays

Ras-binding domain (RBD) protein for active Ras pulldowns was prepared by subcloning human cRaf (a.a. 1–149) into pGEX-5x-3 (GE Healthcare). p21-binding domain (PBD) protein for active RAC pulldowns was prepared by subcloning human PAK1 (a.a. 70–117) into the pGEX2TK vector. Rac1G15A Agarose (Cell BioLabs) was used for active RAC-GEF pulldowns. For a detailed protocol see Supplemental Information.

Primary Acinar Cell Isolation

Pancreata were harvested from 6- to 8-week old mice, washed twice in Hanks balanced salt solution (HBSS), minced and incubated with 0.2 mg/mL collagenase-P (Roche, Indianapolis, IN) at 37°C for 15 min. Tissue was washed 3x in HBSS containing 5% fetal bovine serum (FBS) and filtered through 500µm and 105µm polypropylene mesh (Spectrum Laboratories). Filtrate was centrifuged through 30% FBS in HBSS and resuspended in Waymouth MB 752/1 medium (Sigma-Aldrich) supplemented with 50 µg/mL gentamycin (Life Technologies), 0.4 mg/mL soybean trypsin inhibitor (Life Technologies), and 1 µg/mL dexamethasone (Sigma-Aldrich). Suspension was plated in non-adherent plates and maintained at 37°C in a 5% CO₂ atmosphere. Transdifferentiation assays performed as described⁹.

Adenoviral Infection and Drug Treatment of Primary Acinar Cells

Isolated acinar cells were infected with either adenoviral GFP or adenoviral Cre and maintained at 37°C in a 5% CO₂ atmosphere for 3 days. Cells were washed once in PBS, lysed in MLB buffer containing protease and phosphatase inhibitors and analyzed by immunoblot.

Quantitative RT-PCR

Acinar cells were washed once in PBS, lysed in RLT plus Buffer (Qiagen) and passed 5 times through a 22 gauge needle. RNA was purified using RNeasy plus kit (Qiagen) according to the manufacturer's instructions. All real-time PCR reactions were performed in triplicate. qRT-PCR on mouse acinar cells was performed using Fast SYBR Green master mix (Applied Biosystems) on a ViiA 7 Real-Time PCR system (Applied Biosystems) and analyzed with ViiA 7 version 1.1 software. The relative amount of each gene was normalized to HPRT1 and RPL13A for mouse acinar cells by a $\Delta\Delta C_t$ method. See Supplemental Methods for primer sequences.

RESULTS

PI3K p110 α is required for Kras-induced pancreatic tumorigenesis

The *Kras*^{LSL-G12D/+}; *Pft1a*^{Cre/+} mouse model of pancreas tumorigenesis (referred to hereinafter as *Kras*^{G12D}), displays all stages of pancreatic intraepithelial neoplasia (PanIN) found in human patients²⁶. To determine the role of the two ubiquitously expressed Type I PI3K isoforms, we bred *p110 α* ^{Flox/Flox} and *p110 β* ^{Flox/Flox} mice into the *Kras*^{G12D} background, resulting in concomitant pancreatic *Kras*^{G12D} expression and deletion of the desired PI3K catalytic subunit. Immunoblot analysis of pancreatic extracts showed that

recombination depleted the targeted p110 protein with no effect on the other (Supplemental Fig. 1A). Mice lacking p110 α ($\alpha^{-/-}$) or p110 β ($\beta^{-/-}$) in the pancreas were viable and fertile. Pancreata appeared normal, although p110 α -null pancreata were consistently smaller (Supplemental Fig. 1B and C) due to smaller acinar cells (Supplemental Fig. 1D). *Kras*^{G12D} pancreata showed multiple areas of metaplasia and mPanIN at 8-weeks of age (Fig. 1). However, when p110 α was ablated from *Kras*^{G12D} mice, no lesions were found by gross or histological examination at 8-weeks (Fig. 1) or 16-weeks of age (data not shown). Fibrosis was also muted as determined by Picrosirius red stain. Six 12-month-old $\alpha^{-/-};Kras^{G12D}$ mice also showed no tumors, suggesting that p110 α ablation introduces a long-term blockade in tumor initiation (data not shown). In contrast, pancreas mass increased significantly and tumors formed efficiently in $\beta^{-/-};Kras^{G12D}$ mice (Fig. 1 and Supplemental Fig. 1C), indicating that the p110 β isoform does not play an essential role in pancreatic tumorigenesis. The lack of tumorigenesis was not due to insufficient levels of active RAS as *Kras*^{G12D} and $\alpha^{-/-};Kras^{G12D}$ lysates contained equivalent amounts of GTP-bound RAS (Supplemental Fig. 2A). MAPK activity was also not affected, as indicated by equivalent levels of pERK1/2 in *Kras*^{G12D} and $\alpha^{-/-};Kras^{G12D}$ pancreatic lysates (Supplemental Fig. 2B). A similar tumor-blocking effect resulting from EGF receptor (EGFR) ablation is circumvented by ablating Trp53⁹. However, *p110 α ^{Flox/Flox}* mice crossed into the *Kras*^{LSL-G12D/+}; *Trp53*^{LSL-R172H/+}; *Ptf1a*^{Cre/+} background²⁸ did not develop tumors (Supplemental Fig. 3), indicating that p110 α ablation creates an insurmountable blockade in tumorigenesis.

p110 α ablation and chronic inhibition upregulates AKT signaling

The oncogenic effects of PI3K are generally attributed to its regulation of AKT. However, we find a substantial elevation of phospho-AKT in both $\alpha^{-/-}$ and $\alpha^{-/-};Kras^{G12D}$ pancreatic lysates (Fig. 2A) and high levels in $\alpha^{-/-};Kras^{G12D}$ acinar cells (Fig. 2B), compared to *Kras*^{G12D} pancreata. While pAKT was elevated in $\alpha^{-/-}$ mice compared to control, it was higher still when *Kras*^{G12D} was expressed, demonstrating that *Kras* signals to AKT in the absence of p110 α . To determine if elevated AKT activation was dependent on signaling from other PI3K isoforms, we isolated primary acinar cells from *Kras*^{G12D} and $\alpha^{-/-};Kras^{G12D}$ mice and treated them with GDC0941, a broad spectrum PI3K inhibitor. In cells from both genotypes, GDC0941 eliminated AKT activity (Fig. 2C). This effect was analogous to upregulation of pAKT observed when PDA cell lines from mouse (NB490) and human (MiaPaCa2) were chronically treated with the p110 α selective inhibitor PIK75. With 1 day of treatment, PIK75 reduced pAKT levels (data not shown). However, when treatment was extended to 3 days, pAKT levels increased by 2–3 fold, which was reversed by treatment with GDC0941 (Fig. 2D). Immunoblotting for each p110 isoform in wild-type, *Kras*^{G12D} and $\alpha^{-/-};Kras^{G12D}$ acinar cells revealed that all p110 isoforms were expressed. p110 γ was expressed under all conditions whereas p110 α and p110 β were substantially higher in *Kras*^{G12D} cells (Fig. 2E). We conclude that elevated levels of pAKT upon p110 α ablation results from compensatory activity from other PI3K isoforms. Most importantly, these data revealed that elevated AKT activity is not, by itself, sufficient to initiate pancreatic tumorigenesis.

PI3K p110 α -null acinar cells resist actin cytoskeleton rearrangement and cannot sustain metaplasia

PI3K has been proposed to support PDA progression by promoting acinar cell transdifferentiation to transformation-susceptible metaplastic ducts¹⁸. However, we found that *in vitro* ADM was efficient in primary cultures of acinar cells lacking p110 α (Supplemental Fig. 4). Nevertheless, the complete absence of metaplasia in $\alpha^{-/-};Kras^{G12D}$ pancreata *in vivo* still suggested an effect on metaplastic ducts. To explore this, we examined the effects of acute cerulein treatment, which accelerates tumorigenesis by promoting ADM³¹. $Kras^{G12D}$ and $\alpha^{-/-};Kras^{G12D}$ mice were treated with supraphysiological doses of cerulein sufficient to induce widespread metaplasia and neoplasia in 30-day old $Kras^{G12D}$ mice by 7 days after treatment⁹. With this protocol, $\alpha^{-/-};Kras^{G12D}$ mice showed complete protection from tumorigenesis and metaplasia (Fig. 3). To reconcile this observation with our *in vitro* ADM results, we reduced recovery time and examined the pancreata histologically. With only 1 day of recovery, we found isolated areas of metaplastic tissue, complete with acinar cell dropout, dilated acini and reactive stroma (Fig. 3). CK19 IHC confirmed the identity of bona fide metaplastic ducts within these regions. These data suggested that, together with our *in vitro* results, p110 α activity is not required for metaplastic duct formation, but is instead required for their maintenance *in vivo*.

Given the elevated pAKT levels in the $\alpha^{-/-};Kras^{G12D}$ mice, we asked if compromised RAC1 function could explain the tumorigenesis blockade. We first confirmed that $Rac1^{-/-};Kras^{G12D}$ mice formed few to no tumors with age²⁰, an essential phenocopy of $\alpha^{-/-};Kras^{G12D}$ mice (Supplemental Fig. 5). We then tested if $Rac1^{-/-};Kras^{G12D}$ mice were resistant to cerulein-induced tumorigenesis. Just as in $\alpha^{-/-};Kras^{G12D}$ mice, $Rac1^{-/-};Kras^{G12D}$ mice were completely protected from ADM and tumorigenesis with 7 days recovery, but contained areas of CK19-positive metaplastic tissue with 1 day recovery (Fig. 3).

Inflammation has been shown to be involved in ADM via production of specific cytokines³². To test if the inability to maintain metaplastic structures could be related to the inflammatory response, we stained pancreata from cerulein-treated mice for CD45 (Supplemental Fig. 6). All genotypes showed significant upregulation of inflammatory infiltration upon cerulein treatment. Consistent with a possible role of reduced inflammatory signaling, $\alpha^{-/-};Kras^{G12D}$ mice had significantly fewer CD45 positive cells than $Kras^{G12D}$ mice. However, $Rac1^{-/-};Kras^{G12D}$ mice showed no such reduction, indicating that other cell autonomous mechanisms were likely to be responsible for this specific phenotype.

We then examined actin rearrangement during early tumorigenesis, a process dependent on RAC1 activity²⁰. Taking advantage once again of the acute nature of cerulein-induced tumorigenesis, 30-day old $Kras^{G12D}$ mice were treated with cerulein for 3 days and sacrificed 1 hour after the final cerulein injection to highlight early changes within the acinar cell compartment, prior to significant ADM. Sections were co-stained with phalloidin to monitor the actin cytoskeleton, and anti-ZO-1, to highlight the acinar cell apical surface. In $Kras^{G12D}$ saline-injected controls, phalloidin and ZO-1 strongly co-localized. In cerulein

treated *Kras*^{G12D} pancreata, phalloidin staining highlighted the basolateral surface, indicating active actin cytoskeleton rearrangement (Fig. 4). In contrast, acinar cells in $\alpha^{-/-};Kras^{G12D}$ pancreatic sections resembled saline-treated controls, maintaining phalloidin/ZO-1 colocalization at the apical surface. This was essentially identical to *Rac1*^{-/-};*Kras*^{G12D} pancreata (Fig. 4), where some mobilization to the basolateral surface was apparent, possibly due to activity of other small G-proteins.

Kras signals through p110 α to activate RAC1 early in transformation

The discovery that *Rac1*^{-/-};*Kras*^{G12D} mice strongly phenocopied $\alpha^{-/-};Kras^{G12D}$ mice led us to examine RAC1 activity in $\alpha^{-/-};Kras^{G12D}$ pancreata. Both activated (GTP-bound) and total RAC1 levels were higher in *Kras*^{G12D} pancreas lysates, as compared to wild-type control (Fig. 5A). p110 α ablation significantly diminished both RAC1 activity and expression in *Kras*^{G12D} pancreata (Fig. 5B). PI3K is known to affect RAC1 activity by regulating PIP₃-dependent RAC1 GEF activity³³, which promotes GDP-GTP exchange. To test if this was a mechanism of RAC regulation in the pancreas, we focused on two GEFs that seemed particularly relevant to our system. *Vav1* is ectopically expressed in PDA cells and is necessary for cancer cell viability³⁴. *Tiam1* is a Kras-activated GEF overexpressed in breast and prostate cancers^{35, 36}. We found elevated levels of active and total VAV1 and TIAM1 in *Kras*^{G12D} pancreatic extracts, compared to control (Fig. 5C). Both active and total VAV1 and TIAM1 were significantly lower in $\alpha^{-/-};Kras^{G12D}$ pancreata (Fig. 5C).

To test if p110 α -dependent upregulation of the *Rac1* pathway is present in the earliest stages of tumorigenesis, we examined RAC1 and GEF levels in primary acinar cells isolated from six to eight-week old *Kras*^{G12D} and $\alpha^{-/-};Kras^{G12D}$ mice. Similar to results obtained from whole pancreas lysates, RAC1 activity was elevated in *Kras*^{G12D} acinar cells and p110 α ablation reduced this activity to control levels (Fig. 6A). Consistent with these results, VAV1 expression was higher in *Kras*^{G12D} acinar cells but this increase was significantly attenuated in $\alpha^{-/-};Kras^{G12D}$ samples (Fig. 6A). TIAM1 was not detectable in isolated acinar cells.

To test whether VAV1 expression could be induced acutely upon Kras activation, we isolated acinar cells from *Kras*^{LSL-G12D/+} mice and infected them with adenovirus encoding either Cre recombinase (Ad-Cre) or GFP (Ad-GFP), as a control. 3 days after adenovirus infection, Cre-induced expression of oncogenic *Kras* resulted in upregulation of VAV1 (Fig. 6B). In contrast, in Ad-Cre infected *p110 α ^{flox/flox}; Kras^{LSL-G12D/+}* acinar cells, VAV1 expression was attenuated (Fig. 6B).

Finding that oncogenic Kras expression was sufficient to upregulate VAV1 protein, we then tested if transcripts for *Vav1* and other GEFs were affected. Using qRT-PCR, we found that *Vav1* and *Dock10* mRNA levels were significantly upregulated in *Kras*^{G12D} primary acinar cells. *Rac1* and *Tiam1* mRNA levels were elevated, but not significantly (Fig. 6C). *Vav1*, *Tiam1*, *Dock10* and *Rac1* mRNA expression was not upregulated in $\alpha^{-/-};Kras^{G12D}$ cells (Fig. 6C). Taken together, these results indicate that oncogenic Kras can increase *Rac* pathway transcripts in acinar cells in a PI3K p110 α dependent manner, but this is unlikely to be the major mechanism of *Rac* regulation.

It was surprising to find VAV1 protein in *Kras*^{G12D} acinar cells, since its expression is generally thought to be confined to inflammatory cells, though expression has been observed in PDA³⁴. Vav1 IHC in *Kras*^{G12D} pancreata revealed expression in stromal cells, as expected, but also at very high levels in what appeared to be tuft cells in *Kras*^{G12D} metaplasia and mPanINs³⁰. Tuft cell expression was confirmed by co-IF for Vav1 with phalloidin to highlight their characteristic actin-rich bundles (Fig 6D).

We then examined expression of p110α and components of the RAC pathway for which we could stain by IHC in a human pancreas tissue microarray containing normal, pancreatitis, PanIN and PDA tissue (Figure 6E, Table 1). p110α was detectable in 10/25 of normal pancreas samples, generally at low levels. p110α was detectable in the majority of chronic pancreatitis, PanIN and PDA samples, often at high levels (Table 1, Fig. 6E). As seen in mice, VAV1 expression was limited to tuft cells in normal, pancreatitis and PanIN lesions that reflected the frequency with which tuft cells were found in these sample types^{30, 37}. VAV1 expression in PDA samples was uniform throughout the cancer epithelium (data not shown). TIAM1 was found at high levels in islets in all normal and diseased tissues but at modest levels in normal exocrine cells. TIAM1 was expressed in the epithelium of the majority of pancreatitis and PanIN samples and ~50% of PDAs within tumor epithelium. ADM adjacent to PDA universally expressed elevated TIAM1. Rac1 expression was easily detectable in all normal pancreas, metaplasia, PanINs, and PDAs. RAC1 levels were uniform throughout the epithelia in normal and pancreatitis samples. In contrast, the majority of PanIN lesions showed elevated Rac1 levels, as did about half of PDA samples, but in each case staining within the epithelium became less uniform, with several cells within the lesion being negative.

To test if p110α regulation of RAC1 by GEF regulation extended beyond early tumor progression, we investigated the dependency of RAC activity on p110α in human PDA cell lines. CFPAC-1 and Capan-2 cells have been reported to express VAV1³⁴. Acute knockdown using transient transfection of siRNAs targeting VAV1 or TIAM1 reduced RAC activity in both cell lines (Supplemental Fig. 7A&B). However, p110α siRNA knockdown diminished RAC activity only in Capan-2 (Supplemental Fig. 7A&B). To examine effects of chronic knockdown of p110α, shRNA lentiviruses targeting p110α were stably transfected into Capan-2 and CFPAC-1 cells and transcripts of several GEFs reported to be associated with PI3K, including VAV1, DOCK10, PREX1 and TIAM1, were examined by qRT-PCR. VAV1 was significantly lower in both cell lines with p110α knocked down (Supplemental Fig. 7C), whereas TIAM1 and other GEFs were reduced in one line or the other (Supplemental Fig. 7C) and PREX1 even increased in CFPAC-1 knockdown cells. Together, our data indicate that p110α can continue to affect the RAC pathway in adenocarcinoma, but in a less consistent manner than it does during tumorigenesis.

DISCUSSION

Though previous attempts have proven ineffective, there is renewed interest in targeting oncogenic Kras, the primary initiator of PDA. Complementary to these efforts is the identification of Kras downstream effectors and their precise roles in PDA progression. Here we have conditionally ablated the ubiquitously-expressed PI3K p110 isoforms p110α and

p110 β from a *Kras*^{G12D} pancreatic tumorigenesis model to study the importance of these endogenous effectors. In this system, these isoforms clearly have non-overlapping functions, as p110 α ablation gave complete protection from tumorigenesis whereas p110 β ablation provided none. Moreover, tumorigenesis blockade induced by p110 α ablation was not overcome by introducing a mutant *Trp53* allele. *Trp53* ablation was capable of bypassing a similar level of protection conferred by EGFR deletion^{9, 38}. Unlike EGFR ablation, which results in inhibition of AKT signaling³⁸, p110 α was dispensable for AKT activation by *Kras*^{G12D} *in vivo*. However, p110 α was required for *Kras*-induced activation of RAC1, a known regulator of pancreatic tumorigenesis.

Previous reports have implicated PI3K in PDA^{18, 39}. PI3K p110 γ is overexpressed in human PDA³⁹. Pharmacological and siRNA down-regulation of p110 γ confirms its involvement in proliferation of PDA cell lines³⁹. Recently, it was found that a knock-in of a constitutively active p110 α ^{H1047R} mutant into the ROSA26R locus induces pancreatic tumorigenesis¹⁸. This is in contrast to a knock-in of this mutant into the endogenous *Pik3ca* locus, which produced no pancreatic abnormalities¹⁹. While these conflicting results make it unclear whether activation of endogenous p110 α is sufficient for pancreatic tumorigenesis, our data establish that it is necessary.

Saur and colleagues clearly establish the necessity of PDK1, the PI3K effector responsible for activating AKT, as genetic ablation of PDK1 blocks *Kras*-induced pancreatic tumorigenesis¹⁸. However, while p110 α ablation completely protected mice from *Kras*-induced tumorigenesis, it did not block AKT activation by *Kras*^{G12D}. Elevated AKT activity in p110 α null pancreata resulted from compensation by other p110 isoforms, and clarifies that AKT activation is not the sole critical function of p110 α in pancreatic tumorigenesis. A similar compensation could be induced with chronic inhibition of p110 α in PDA cell lines, demonstrating that selective inhibition of this PI3K isoform may prove problematic.

Prior studies show that neoplastic lesions in the pancreas arise from acinar cells that have undergone ADM, a reprogramming event mediated by EGFR signaling⁴⁰. Given the complete absence of metaplasia and neoplasia, we were surprised to find that ablation of p110 α did not block ADM *in vivo* or *in vitro*. However, without p110 α , *Kras*^{G12D} was not capable of stabilizing and transforming ADM after cerulein stimulation, resulting in restoration of apparently normal pancreatic tissue given sufficient recovery time. Saur and colleagues show broad spectrum PI3K inhibitors can block ADM *in vitro*¹⁸. We find that use of a relatively selective p110 α inhibitor PIK75 is ineffective at blocking *in vitro* ADM until used at a nonselective concentration (data not shown). Together, these results suggest that AKT activity is critical for *in vitro* ADM and the higher levels of pAKT after p110 α ablation is sufficient to induce the process.

Finding that p110 α was important for actin rearrangement in *Kras*^{G12D} expressing acinar cells, we focused on regulation of RAC1, which is critical for this process²⁰. Siveke and colleagues show that RAC1 expression increases in *Kras*-induced pancreas tumors, and ablation of *Rac1* significantly reduces *Kras*-induced pancreatic tumorigenesis²⁰. We found that deletion of p110 α in *Kras*^{G12D} mice led to decreased RAC1 expression and, more importantly, RAC1 activity, in pancreas tissue and acinar cells. It is well-established that

several RAC GEFs contain PH domains and thus can be activated and organized within PIP₃ containing membranes. We found that the activities of the RAC-GEFs VAV1 and TIAM1 were highly dependent on p110 α (Fig. 5C) and these GEFS were also frequently expressed in precancerous lesions in humans (Fig. 6E). Taken together, our data suggest that p110 α regulation of PH-domain-containing RAC GEFs may collectively contribute to tumor development.

In summary, we have shown that PI3K p110 α is required for Kras-induced transformation of ADM via its regulation of RAC1 and could represent a target for PDA therapy. Indeed, a pan Class I PI3K inhibitor, GDC0941, effectively blocks tumor growth in *Kras*^{G12V};*P53*^{R172H};*Ptfla*-*Cre* mice¹⁸. However, pan-PI3K inhibitors that target both p110 α and p110 β could have unacceptable cardiac toxicities⁴¹. While we see inhibition of growth with acute treatment of PDA cell lines with PIK75, within days of treatment, cells began to become resistant (data not shown) in a time frame consistent with the observed upregulation of pAKT (Fig. 2D). Similarly, we observed no changes in cell proliferation or apoptosis after PIK75 treatment of established tumors in *Kras*^{G12D} mice (data not shown), also possibly due to compensation by other PI3K isoforms. We conclude that while p110 α regulation of RAC1 is required for tumorigenesis, the maintenance of AKT activity is compensated for by other p110 family members, suggesting its promise as a drug target for PDA treatment likely lies in optimized combination therapies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Ablation of class IA PI3K p110 α , but not p110 β , blocks Kras-induced tumor formation
 Spontaneous mPanIN formation in 8-week old *Kras*^{G12D}, $\alpha^{-/-};Kras^{G12D}, and $\beta^{-/-};Kras^{G12D} shown by hematoxylin and eosin staining (H&E), dual IHC for the ductal marker CK19 (brown) and the acinar marker amylase (blue) and picrosirius red stain (Sirius red). Scale bar=100 μ m.$$

Fig. 2. pAkt is elevated in p110α-null pancreata

(A) Pancreas extracts from WT, $\alpha^{-/-}$, $Kras^{G12D}$, and $\alpha^{-/-};Kras^{G12D}$ were analyzed by immunoblot with the indicated antibodies. (B) IHC for phospho-AKT(T308) on sections from $Kras^{G12D}$, and $\alpha^{-/-};Kras^{G12D}$ pancreata. Scale bar=100μm for panels and 50μm for insets. Metaplasia (M) and mPanIN (P) are indicated. (C) Isolated acinar cells from the indicated genotypes were treated with DMSO or the pan PI3K inhibitor GDC0941 (500nM) for 1 hour. Total cell lysates were analyzed by immunoblot for AKT and p-AKT (T308). (D) MiaPaCa2 and NB490 cells were treated with either DMSO or the p110α selective inhibitor PIK75 (50nM) for 3 days followed by treatment with the pan-p110 inhibitor GDC0941 (500nM) for 1 hour. Cell lysates were analyzed by immunoblot for AKT and p-AKT(T308). Numbers indicate densitometric quantitation of p-AKT/AKT ratios. (E) Immunoblots for the indicated p110 isoforms in lysates from wild-type (WT), $Kras^{G12D}$, and $\alpha^{-/-};Kras^{G12D}$ acinar cells.

Fig. 3. PI3K p110 α -null or Rac1-null acinar cells cannot sustain ductal metaplasia
Kras^{G12D}, $\alpha^{-/-};Kras^{G12D}$, and *Rac1^{-/-};Kras^{G12D}* mice were treated once daily for 5 days with cerulein and allowed to recover for 1 day or 7 days. Sections were H&E stained and IHC was performed for CK19 (inset). Asterisks indicate areas depicted in insets. Scale bar=100 μ m for panels, 32.5 μ m for insets.

Fig. 4. Actin cytoskeleton rearrangement requires both p110 α and Rac1

Immunofluorescence staining for ZO-1 (green) and phalloidin (red) on saline-treated *Kras*^{G12D} and cerulein-treated *Kras*^{G12D}, $\alpha^{-/-}$; *Kras*^{G12D}, and *Rac1*^{-/-}; *Kras*^{G12D} mice. Mice were treated once daily for 3 days and sacrificed one hour following the last injection. Nuclei are counter-stained with DAPI. Scale bar=20 μ m. Graph shows quantitation of phalloidin and ZO-1 colocalization coefficients for the indicated genotypes. Values are means $\pm$ S.E.M. of 10 fields of view from 3 mice of each genotype or treatment regimen as indicated. Significance was determined by one-way ANOVA.

Fig. 5. Kras signals through p110 α to activate Rac1 in the early stages of transformation
 (A) RAC1 activity was measured in total lysates from WT and *Kras*^{G12D} pancreata by PAK-PBD pulldown and total RAC1 levels were measured by immunoblot. Protein loading was assessed by ponceau stain (Loading Control). Numbers indicate densitometric quantitation normalized to loading control (B) Total lysates from *Kras*^{G12D} and $\alpha^{-/-}; Kras^{G12D} pancreata were assessed for RAC1 activity by PAK-PBD pulldown. RAC1 levels in the lysate were measured by immunoblotting for RAC1 with GAPDH as a loading control. Numbers indicate densitometric quantitation normalized to the GAPDH. (C) Total lysates from WT, $\alpha^{-/-}$, *Kras*^{G12D}, and $\alpha^{-/-}; Kras^{G12D} pancreata were analyzed by the Rac1G15A pulldown to measure VAV1 and TIAM1 activities. Levels of VAV1 and TIAM1 in lysates were analyzed by immunoblot. HSP90 was the loading control. Numbers indicate densitometric quantitation normalized to HSP90.$$

Fig. 6. Kras increases RAC-GEF expression in a p110α dependent manner

(A) RAC1 activity was measured in WT, $Kras^{G12D}$, and $\alpha^{-/-};Kras^{G12D}$ acinar cell lysates by PAK-PBD pulldown compared to total VAV1, RAC1, and GAPDH levels. Numbers indicate densitometric quantitation normalized to GAPDH. (B) Acinar cells from $Kras^{LSL-G12D/+}$ and $p110\alpha^{Flox/Flox};Kras^{LSL-G12D/+}$ mice (without *Ptf1a-Cre*) were isolated, cultured, and infected with either adeno-GFP or adeno-Cre for 3 days. Lysates were assayed for levels of VAV1 and ACTB by immunoblotting. Numbers indicate densitometric quantitation normalized to ACTB. (C) Quantitative RT-PCR for *Vav1*, *Tiam1*, *Dock10*, and *Rac1* was performed mRNA isolated from WT, $Kras^{G12D}$, and $\alpha^{-/-};Kras^{G12D}$ acinar cells. Values are means $\pm$ S.E.M. of fold change compared to WT from 5 independent experiments. Significance was determined by one-way ANOVA. (D) 4-month old $Kras^{G12D}$ pancreata were stained for VAV1 (left panel). Scale bar=100μm. Immunofluorescence for Vav1 (green) and phalloidin (red) was performed on frozen sections from 4 month old $Kras^{G12D}$ mice (right panel). Nuclei were counterstained with DAPI (blue). Scale bar = 20μm. (E) Representative images from human TMA slides stained for p110α, VAV1, TIAM1 or RAC1 in normal pancreas (top panels) and PanINs (middle and bottom panels). Scale bar=100μm for panels, 50μm for insets.

Table 1

Summary of TMA IHC for RAC1 Pathway components

Human pancreas tissue microarrays were stained for p110α, VAV1, TIAM1 or RAC1. Expression was scored as undetectable (0), detectable (1) or high expression (2) in normal tissue, chronic pancreatitis, PanIN1, PanIN2, PanIN3 and pancreatic ductal adenocarcinoma (PDA). (TC) indicates that expression is limited to tuft cells.

	Normal Pancreas			Pancreatitis			PanIN1			PanIN2			PanIN3			PDA		
	0	1	2	0	1	2	0	1	2	0	1	2	0	1	2	0	1	2
p110α	15	10	-	13	14	12	2	1	8	3	5	9	1	1	6	5	14	11
VAV1	23	-	2(TC)	26		13(TC)	3	-	8(TC)	4	-	13(TC)	6	-	2(TC)	23	-	7
TIAM1	-	25	-	6	18	15	1	2	7	-	5	12	1	-	7	12	13	5
RAC1	-	25	-	-	36	3	-	5	6	-	8	9	-	1	7	-	17	13