Selective Requirement of PI3K/PDK1 Signaling for Kras Oncogene-Driven Pancreatic Cell Plasticity and Cancer

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SUMMARY

Oncogenic Kras activates a plethora of signaling pathways, but our understanding of critical Ras effectors is still very limited. We show that cell-autonomous phosphoinositide 3-kinase (PI3K) and 3-phosphoinositide-dependent protein kinase 1 (PDK1), but not Craf, are key effectors of oncogenic Kras in the pancreas, mediating cell plasticity, acinar-to-ductal metaplasia (ADM), and pancreatic ductal adenocarcinoma (PDAC) formation. This contrasts with Kras-driven non-small cell lung cancer, where signaling via Craf, but not PDK1, is an essential tumor-initiating event. These in vivo genetic studies together with pharmacologic treatment studies in models of human ADM and PDAC demonstrate tissue-specific differences of oncogenic Kras signaling and define PI3K/PDK1 as a suitable target for therapeutic intervention specifically in PDAC.

INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC) is nearly uniformly fatal despite maximal treatment, with fewer than 1% of patients surviving 5 years (Carpelan-Holmström et al., 2005). A wealth of molecular studies have identified mutant Kras as the initiating event (Hidalgo, 2010; Hingorani et al., 2003; Morris et al., 2010; Pinho et al., 2011; Seidler et al., 2008). Oncogenic Kras activates a plethora of signaling pathways, including canonical Raf/MEK/ERK, PI3K/AKT, RalGDS/p38 MAPK, Rac and Rho, Rassf1, NF1, p120GAP, and PLC-ε (Castel-lano and Downward, 2011; Pylayeva-Gupta et al., 2011). However, which of these effector pathways of oncogenic Kras control cell fate decisions and PDAC formation remains an outstanding question (Morris et al., 2010; Pylayeva-Gupta et al., 2011). The PI3K/AKT pathway is uniformly activated in human PDAC and mouse models of Kras-driven pancreatic cancer

Significance

Kras-driven tumors such as PDAC, NSCLC, or colon cancer differ in prognosis and response to targeted therapies. However, the underlying molecular mechanisms are largely unknown. Oncogenic Kras activates diverse signaling pathways but the functional relevance of specific Kras effectors is unclear for most cancer types. Because Kras is considered undruggable, this has hampered progress toward targeted therapeutic interventions. We provide in vivo genetic evidence for context-specific effector pathways of oncogenic Kras in PDAC and NSCLC and define cell autonomous Kras \( \rightarrow \) PI3K \( \rightarrow \) PDK1 signaling as a critical and therapeutically tractable axis in pancreatic cancer initiation and maintenance.
Expression of Oncogenic p110α,

RESULTS

despite the contributions of Kras effector pathways to PDAC and NSCLC patient-derived humanized cancer models, we set out to analyze expression of the ubiquitously expressed class IA p110α. Hotspot mutations of p110α that activate PI3K signaling have been identified in the helical domain (E542K and E545K) and the catalytic domain (H1047R) in a variety of human cancers, including breast and lung (Bader et al., 2005; Liu et al., 2009). Transgenic expression of p110αH1047R in mice induces breast and lung cancer (Adams et al., 2011; Engelman et al., 2008; Liu et al., 2011).

Taking advantage of genetically engineered murine and patient-derived humanized cancer models, we set out to analyze the contribution of Kras effector pathways to PDAC and NSCLC development.

Pancreas-Specific PI3K Pathway Activation by Expression of Oncogenic p110αH1047R Induces ADM and Premalignant PanIN

To explore the role of the PI3K/AKT signaling pathway in Kras-induced cell plasticity, ADM, and PDAC development, we generated a latent oncogenic PIK3CAH1047R (encoding p110αH1047R) allele silenced by a lox-stop-lox (LSL) cassette as a knock-in mouse (Figures S1A–S1C available online). To activate the expression of p110αH1047R and thus PI3K signaling, specifically in the pancreas, we used the well-established Ptf1aCre+ driver line to direct recombination in pancreatic acini, ducts, and islets (Figures S1D and S1E) (Nakahai et al., 2007; Seidler et al., 2008; von Werder et al., 2012).

Transgenic expression of p110αH1047R from the Rosa26 locus resulted in moderately increased PI3P levels in the pancreas, similar to expression of KrasG12D from the endogenous Kras locus in the established KrasG12D knock-in mouse model (Figures 1A and 1B). Importantly, we observed no obvious difference in the p110α protein levels in pancreatic tissue lysates between Ptf1aCre+/LSL-PIK3CAH1047R/+ animals and Ptf1aCre+/LSL-KrasG12D knock-in mice (Figure 1B), even though Ptf1aCre+/LSL-PIK3CAH1047R/+ animals carried PIK3CAH1047R in the Rosa26 locus in addition to their endogenous Pik3ca. Ptf1aCre+/LSL-PIK3CAH1047R/+ mice were viable and revealed no overt phenotype after birth. However, pancreatic size and weight were increased, as previously shown in the KrasG12D model (Figure 1C).

Histopathologic analyses revealed that all Ptf1aCre+/LSL-PIK3CAH1047R/+ mice developed massive induction of ADM, a condition where terminally differentiated acinar cells dedifferentiate into a progenitor-like state and acquire features of ductal cells (Figure 1D) (Pinho et al., 2011). Furthermore, all animals developed PanIN, a precursor lesion of PDAC (Figure 1D) (Hruban et al., 2006). The amount and grade of these lesions increased over time from PanIN-1A, already present in 1-month-old mice, to PanIN-3, found in some 9-month-old animals, which represents carcinoma in situ (Figures 1D and 1E). The PIK3CAH1047R/+ and KrasG12D/+ models showed very similar patterns of ADM induction and PanIN progression (Figures 1D and 1E) and markers of PI3K pathway activation were almost identical, as shown by immunohistochemistry and western blot analysis of tissue lysates (Figures 1F and 1G). Indicators of PI3K signaling, such as proliferation of PanIN lesions, were consistently similar in both models (Figures S1F and S1G). These observations support the view that pancreas-specific activation of PI3K signaling phenocopies KrasG12D-induced cellular plasticity and PanIN formation.

To confirm that acinar cell plasticity via ADM is indeed involved in PanIN and PDAC development, we used an elastase-1 Cre driver line (Stanger et al., 2005) to specifically activate p110αH1047R in acinar cells. This induced ADM and PanIN lesions with the same frequency as expression of oncogenic KrasG12D (Figures S1H–S1J). In contrast, pancreas-specific constitutive activation of Rac1 signaling by expressing a dominant active Rac1G12V allele from the Rosa26 locus (Ptf1aCre+/LSL-Rac1G12V/+ mice) (Srinivasan et al., 2009) failed to induce ADM and PanINs (Figure S1K). Since Rac1 is a downstream effector of PI3K and is activated in the KrasG12D model of pancreatic cancer (Heid et al., 2011), failure of Rac1G12V to induce ADM and PanINs argues for the specific involvement of canonical PI3K/AKT signaling in pancreatic tumor formation.

Expression of Oncogenic p110αH1047R in the Pancreas Phenocopies KrasG12D-Induced Metastatic PDAC

To test whether p110αH1047R is also capable of inducing pancreatic cancer, we aged Ptf1aCre+/LSL-PIK3CAH1047R/+ mice. All animals in the tumor watch cohort developed PDAC within 800 days (Figures 2A, 2B, and S2A). Comparison of tumor formation and survival of PIK3CAH1047R and KrasG12D mutant animals revealed striking similarities, with nearly identical survival times and similar rates of metastasis (Figures 2A and S2B). p110αH1047R-induced tumors were histopathologically indistinguishable from human and murine KrasG12D-induced PDAC and showed the full spectrum of the human disease, ranging from well-differentiated ductal PDAC to undifferentiated tumors and typical metastasis to lymph nodes, liver, and lung (Figures 2B, S2A, and S2B) (Hruban et al., 2006).

To explore activation of PI3K signaling in p110αH1047R- and KrasG12D-driven pancreatic cancer, we analyzed tissues from these animals using phospho-specific antibodies. Similar activation of the key downstream effectors of PI3K signaling, pAKT-T308, pAKT-S473, and pGSK3β-S9 was observed in both models (Figures 2C and 2D). Importantly, we found that mutant p110αH1047R did not activate Ras in primary tissue specimens and PDAC cell lines from Ptf1aCre+/LSL-PIK3CAH1047R/+ animals, indicating that the effects observed in the PIK3CAH1047R model were not due to Ras cross activation (Figures S2C and S2D). Consistent with data from pancreatic tissues of the Ptf1aCre+/LSL-PIK3CAH1047R/+ model (Figure 1B), we found no obvious increase of p110α expression levels in PIK3CAH1047R mutant PDAC cell lines (Figure S2D). This accords with previous findings that p110α is not bound to the p85 regulatory subunit and rapidly degraded (Engelman et al., 2008).
Figure 1. Constitutive Activation of PI3K Signaling Causes ADM and Neoplastic Changes in the Pancreas

(A) Genetic strategy used to activate p110αH1047R or KrasG12D expression in the pancreas.

(B) Immunoblot analysis of p110α expression levels (upper panel) and PIP3 activity (lower panel) in pancreata of 6-week-old control (Ctrl), Ptf1aCre+/LSL-KrasG12D/+, and Ptf1aCre+/LSL-PIK3CAH1047R/+ compound mutant mice (n = 3 per genotype).

(C) Timeline of pancreas weight (mg) in mice of the different genotypes.

(D) H&E and alcian blue staining of pancreata from mice of the different genotypes at 9 months. PanIN indicates pancreatic intraepithelial neoplasia.

(E) Bar graph showing the number of lesions per genotype at different time points.

(F) Immunohistochemistry for pAKT, pS473, and pGSK3β in pancreata of the different genotypes.

(G) Immunoblot analysis of PI3K, PIP3, and AKT signaling proteins in pancreata of the different genotypes.
Oncogetic PI3K Signaling Activates a Senescence Program in the Pancreas that Is Bypassed by Loss of Cdkn2a

We next analyzed tumor-suppressive mechanisms in the PIK3CA_H1047R model. Expression of p110α_H1047R-induced a senescence program in the pancreas. All low-grade PanIN lesions examined stained positive for senescence-associated β-galactosidase (SA-β-Gal), which has been recently shown to be the only reliable oncogene-induced senescence biomarker in the pancreas (Figure S2E) (Caldwell et al., 2012). Importantly, these lesions showed concomitant upregulation of both Cdkn2a gene products, p16/Ink4a and p19/Arf, and activation of the p53/p21 Cip1 pathway (Figures S2E and S2F).

In PDAC however, p16/Ink4a and p19/Arf expression is lost as demonstrated previously in the KrasG12D model (Figure S2F and data not shown) (Bardeesy et al., 2006). Consistent with this, genomic analyses revealed frequent deletion of the Cdkn2a locus in p110α_H1047R-induced cancers (data not shown). Again, these observations demonstrate that the PIK3CA_H1047R model phenocopies KrasG12D-induced PDAC with respect to tumor suppressor usage (Bardeesy et al., 2006). Accordingly, mimicking loss of heterozygosity of Cdkn2a by inactivation of one allele using floxed Cdkn2a mice (Bardeesy et al., 2006) accelerated PDAC formation in the PIK3CA_H1047R and KrasG12D model (Figures S2G and S2H). To investigate the effect of Cdkn2a inactivation on oncogene-induced senescence of PanIN lesions, we inactivated both Cdkn2a alleles. As expected, this completely...
blocked senescence of early PanIN lesions (Figures S2I and S2J). These findings demonstrate the importance of the Cdkn2a tumor suppressor locus for PDAC progression. Interestingly, median survival times were indistinguishable between both heterozygous deletion models, arguing that identical pathways and tumor suppressors operate in p110αH1047R- and Kras G12D-driven PDAC formation.

Taken together, these murine in vivo modeling studies clearly demonstrate that PI3K signaling induces acinar cell plasticity, ADM, PanIN formation, senescence via upregulation of p16/Ink4a and p19/Arf, bypass of senescence by inactivation of Cdkn2a, and ultimately PDAC formation.

**PI3K Pathway Activation in Human ADM, PanIN, and PDAC**

PI3K/AKT activation is a classical and uniform feature of human PDAC (Jimeno et al., 2008; Kennedy et al., 2011; Reichert et al., 2007; Ying et al., 2011). However, the role of PI3K/AKT signaling during the early stages of human pancreatic carcinogenesis, namely ADM and PanIN formation, remains unclear. To test our hypothesis that the PI3K/AKT pathway is activated in human ADM, PanIN and PDAC, we analyzed key surrogates of PI3K signaling: AKT-T308/S473 and GSK3β-S9 phosphorylation (Figures 3A and 3B). In accordance with the murine in vivo studies, we observed strong activation of PI3K signaling in tissue microarrays of nearly all human ADM (n = 21), PanIN (n = 32), and PDAC (n = 205) specimens (Figure 3C). In addition, we validated PI3K pathway activation in human PDAC using patient-derived primary xenografted tumors, early-passage cell lines, and normal pancreatic tissue (Figure 3D). These data suggest that PI3K/AKT signaling is activated at the earliest stages of tumor evolution in humans and controls pancreatic cell plasticity and carcinogenesis.

**Elimination of PDK1 Invariably Blocks Kras-Driven ADM, PanIN, and PDAC In Vivo**

PI3K activates various downstream effectors by converting phosphatidylinositol (4,5)-bisphosphate (PIP2) into the second messenger PIP3. PIP3 transmits PI3K signals by directly binding to proteins with pleckstrin homology (PH) domains, such as PDK1 and AKT (Cantley, 2002), targeting them to the cell membrane. In turn, PDK1 activates AKT by threonine 308 phosphorylation (Alessi et al., 1997; Currie et al., 1999).

To test whether cell autonomous signaling of the direct PI3K downstream target PDK1 is essential for Kras-driven pancreatic plasticity and PDAC formation, we inactivated Pdk1 specifically in the epithelial compartment of the pancreas using floxed Pdk1
Context-Specific Kras Signaling in PDAC and NSCLC

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KrasG12D-induced pancreatic carcinogenesis, we investigated whether PDK1 substrates other than AKT play a role in probably not directly controlled by PI3K (Pearce et al., 2010). To test our hypothesis, PDK1 inactivation completely blocked PanIN and PDAC formation in the KrasG12D model (Figures 4A–4D). Pancreata showed normal weight and morphology with some areas of fatty degeneration in older animals, a common sign of cellular stress due to Kras oncogene expression (Figures 4B and 4C). Inactivation of PDK1 resulted in normal life expectancy in the KrasG12D model (Ptf1aCre/+;LSL-KrasG12D/+;Pdk1f/f), whereas deletion of one Pdk1 allele (Ptf1aCre/+;LSL-KrasG12D/+;Pdk1f/f) did not alter PanIN and PDAC formation (Figures 4B, 4D, 4F, and S3G). Loss of epithelial PDK1 expression in the KrasG12D model correlated well with PI3K/AKT pathway inactivation (Figures 4E, 4F, and S3I) without affecting Ras activity (Figure 4G) or PIP3 levels (Figure S3F). Importantly, we did not observe hypoplasia or developmental defects of the pancreas, pancreatic islets, and beta cells, or overt hyperglycemia and lethality due to PDK1 inactivation in pancreas epithelia as reported by other groups using different Cre-driver lines and genetic backgrounds (Figures 4B, 4D, S3D, S3G, and S3H) (Hashimoto et al., 2006; Westmoreland et al., 2009). We did however observe impaired glucose tolerance, but this did not progress to diabetes mellitus (Figures S3D and S3H).

Besides AKT, PDK1 also transmits PI3K-dependent signals to SGK and S6K. The PDK1 effectors RSK and isoforms of PKC are not progress to diabetes mellitus (Figures S3D and S3H). It has recently been shown that Craf is essential for KrasG12D-induced NSCLC (Bischof et al., 2011; Karreth et al., 2011). To investigate the contribution of Craf in pancreatic carcinogenesis, we inactivated Craf in pancreas epithelium using floxed Craf mice (Jesenberger et al., 2001). Ptf1aCre/+–induced deletion of Craf in the KrasG12D–driven PDAC model had no inhibitory effect on tumor development or progression and did not improve mouse survival (Figures 6A–6C). Efficient deletion of both Craf alleles and loss of Craf protein expression was verified by genotyping PCR, immunohistochemistry and western blot analysis of PDAC cells isolated from tumor specimens from Ptf1aCre/+;LSL-KrasG12D/+;Craf1f/f mice (Figures 6D–6F). The possibility that tumors developed due to incomplete Craf deletion can thus be excluded. Signaling via Craf is therefore dispensable for initiation of Kras-driven PDAC, supporting the view that Kras exerts its oncogenic effects in a tissue-specific manner.

PIK3CAH1047R/+;KrasG12D/+ double mutant mice (Figures S4A and S4B). Deletion of Pdk1 in both models led to complete inhibition of ADM, PanIN formation, and PDAC development, indicating that Pdk1 is indeed a central node and essential for pancreatic carcinogenesis in diverse in vivo models (Figures S4A and S4B). In contrast, deletion of Pdk1 in KrasG12D–driven NSCLC models had no effect on lung tumor formation (Figures S4C–S4F). Although both the KrasG12D–driven lung and pancreatic cancer models are on a similar genetic background, it remains possible that subtle differences in the genetic background may affect KrasG12D signaling and engagement of PDK1. We therefore used the elastase-1 Cre driver line that recombines loxP sites in the pancreas and the lung of the same animal (Figures 5A–5G). Simultaneous KrasG12D expression in both organs of the same animal induced invasive grade 4 NSCLC and pancreatic tumorigenesis (Figure 5D). Pdk1 deletion blocked pancreatic neoplasia completely, whereas NSCLC formation in the same animal was unaffected (Figures 5E and 5F). Importantly, Pdk1 deletion did not affect overall survival in this model. All animals in the tumor watch cohort developed grade 3–4 NSCLC within 600 days (Figure 5F) and the number of lung lesions was comparable (Figure 5G). These in vivo findings support the view that each tissue has its own unique signaling requirement during Kras oncogene-induced transformation.

Craf Is Dispensable for Kras-Driven PDAC Formation

To test our hypothesis that PI3K/PDK1 signaling transmits oncogenic KrasG12D–induced fail-safe mechanisms, we investigated regulation of PI3K/Ink4, and p19Arf in PanIN and p19Arf in pancreata in which the PI3K signaling pathway had been disrupted (Ptf1aCre/+;LSL-KrasG12D/+;Pdk1f/f model, Figure 4F). PDK1 deficiency significantly reduced p16Ink4a and p19Arf induction compared to Ptf1aCre/+;LSL-KrasG12D/+ animals (Figure 4H). These in vivo genetic studies therefore support the view that KrasG12D–dependent oncogenic stress fluxes through PDK1 to induce upregulation of the p16Ink4a and p19Arf tumor suppressors.

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Disruption of PDK1 or Inhibition of PI3K Signaling Blocks Murine and Human ADM In Vitro

To gain insight into the cellular mechanisms of KrasG12D–PI3K/PDK1–induced transformation, we analyzed early events of pancreatic carcinogenesis. As shown in Figure 4, deletion of Pdk1 blocks not only PanIN development, but also ADM in the KrasG12D model. ADM has recently been suggested to be an initiating event in human and murine PDAC formation (Aicher et al., 2012; Caldwell et al., 2012; Morris et al., 2010; Reichert and Rustgi, 2011). Previous studies demonstrated that transforming growth factor α (TGF-α)/epidermal growth factor receptor and Ras activation induces ADM in vitro (Means et al., 2005; Morris et al., 2010; Reichert and Rustgi, 2011).

PDK1 Is Essential for Kras-Induced Upregulation of p16Ink4 and p19Arf

To test our hypothesis that PI3K/PDK1 signaling transmits oncogenic KrasG12D–induced fail-safe mechanisms, we investigated regulation of PI3K/Ink4, and p19Arf in pancreata in which the PI3K signaling pathway had been disrupted (Ptf1aCre/+;LSL-KrasG12D/+;Pdk1f/f model, Figure 4F). PDK1 deficiency significantly reduced p16Ink4a and p19Arf induction compared to Ptf1aCre/+;LSL-KrasG12D/+ animals (Figure 4H). These in vivo genetic studies therefore support the view that KrasG12D–dependent oncogenic stress fluxes through PDK1 to induce upregulation of the p16Ink4a and p19Arf tumor suppressors.

Elimination of PDK1 Blocks PDAC, but not NSCLC Formation

We next evaluated the role of cell autonomous PDK1 signaling in pancreatic carcinogenesis in PIK3CAH1047R/+ single and
Figure 4. Epithelial PDK1 Is Essential for KrasG12D-Driven Pancreatic Carcinogenesis

(A) Genetic strategy used to study the cell-autonomous role of the PI3K substrate PDK1 in KrasG12D-driven pancreatic cancer formation.

(B) Representative H&E stains of control (Ptf1aCre+;LSL-KrasG12D+) and conditional Pdk1 knockout (Ptf1aCre+;LSL-KrasG12D+/Pdk1f/f) mice.

(C) Pancreatic weight of 6-month-old mice with the indicated genotypes (n.s., not significant; **p < 0.01, Student’s t test).

(D) Kaplan-Meier survival analysis of the indicated conditional genotypes. + denotes the wild-type allele, f the conditional allele (n.s., not significant; ***p < 0.001, log-rank test).

(E) Immunoblot analysis of PI3K/AKT pathway activation in pancreata of 12-month-old Ptf1aCre+;LSL-KrasG12D+ and Ptf1aCre+;LSL-KrasG12D+/Pdk1f/f compound mutant mice.

(F) Immunohistochemical analysis of PI3K/AKT pathway activation in the pancreas of 12-month-old mice with the indicated genotypes. Arrowheads indicate ADM and arrows PanIN-1 lesions.

(G) Analysis of activated Ras (Ras-GTP) in the pancreas of 1- and 12-month-old Ptf1aCre+;LSL-KrasG12D+ and Ptf1aCre+;LSL-KrasG12D+/Pdk1f/f mice.

(H) qRT-PCR analysis of p16/Ink4a and p19/Arf mRNA expression in the pancreas of mice with the indicated genotypes. Data are shown as fold change versus Ctrl. Insets show representative histology in high magnification. Scale bars, 50 μm for micrographs, 20 μm for insets. Error bars, ± SEM.

See also Figure S3.
Figure 5. PDK1 Is Essential for KrasG12D-Driven PDAC but not NSCLC Formation

(A) Genetic strategy used to analyze Ela1-CreERTM mediated recombination in the lung and the pancreas of the same animal in the absence of tamoxifen with a double fluorescent floxed tdTomato-EGFP reporter line (R26mT/mG). (B and C) Confocal microscopic images of tdTomato (red) and Cre-induced EGFP (green) expression in the lung (B) and pancreas (C) of Ela1-CreERTM;R26mT/mG (left panel) and Ctrl R26mT/mG (right panel) animals. Nuclei were counterstained with TO-PRO-3 (blue). Note the constitutive Cre activity in the pancreas and lung in the absence of tamoxifen in Ela1-CreERTM animals.

(D) Genetic strategy used to activate Kras G12D in the lung and the pancreas of the same animal (left panel). H&E stained representative microscopic lung sections graded according to the established 4-stage NSCLC grading system from Ela1-CreERTM;LSL-KrasG12D/+ mouse (upper right panel). Lower right panel: Representative H&E or alcian blue (AB) stained microscopic pancreas sections from the same animal. Activation of oncogenic Kras G12D in lung and pancreas using the Ela1-CreERTM driver line induces grade 4 NSCLC and pancreatic neoplasia.

(E) Genetic strategy used to study the role of PDK1 in KrasG12D-driven lung and pancreatic cancer formation in the same animal (left panel). Representative H&E stained microscopic lung sections graded according to the established 4-stage NSCLC grading system from Ela1-CreERTM;LSL-KrasG12D/Pdk1f/f mouse (upper right panel). Lower right panel: Representative H&E stained microscopic pancreas sections from the same animal. Deletion of Pdk1 blocks ADM and PanIN formation in the pancreas completely but has no effect on NSCLC development and progression.

(F) Kaplan-Meier survival curves of the indicated genotypes (n.s., not significant; ***p < 0.001, log-rank test). Note: All Ela1-CreERTM;LSL-KrasG12D/+ and Ela1-CreERTM;LSL-KrasG12D;Pdk1f/f animals developed NSCLC.

(G) Quantification of microscopic lung lesions of Ela1-CreERTM;LSL-KrasG12D/+ and Ela1-CreERTM;LSL-KrasG12D;Pdk1f/f mice (n.s., not significant, Student’s t test). Insets show representative lesions in high magnification. Scale bars, 50 μm for micrographs, 20 μm for insets. Note: All Ela1-CreERTM animals were analyzed without Cre activation due to constitutive Cre activity (no tamoxifen treatment). Error bars, ± SEM.

See also Figure S4.
To test whether TGF-α/Kras-induced ADM formation depends on intact PDK1 signaling, we isolated acini from Ptf1aCre/+;KrasG12D/+ and Ptf1aCre/+;KrasG12D/+;Pdk1f/f pancreata and performed an in vitro ADM assay (Means et al., 2005). Consistent with the in vivo data (Figures 7A and 7B), we observed that deletion of Pdk1 completely blocked ADM in the presence and absence of TGF-α (Figures 7C and 7D). Acini isolated from Ptf1aCre/+;KrasG12D/+ and Ptf1aCre/+;KrasG12D/+;Pdk1f/f pancreata were equally viable (data not shown), thus excluding differences in cellular vulnerability as a possible cause. ADM was also blocked by the pan class I PI3K inhibitor GDC 0941, the PDK1 inhibitor BX912, the dual pan class I PI3K-mTOR inhibitor NPV-Bez235, and the AKT inhibitor MK-2206 (Figure 7E). Interestingly, the RSK inhibitor BI-D1870 had no effect on ADM formation even at concentrations as high as 10 μM (Figure 7E). These data confirm our in vivo genetic studies in mice and indicate that disruption of the canonical PI3K-PDK1-AKT, but not the PDK1-RSK axis, blocks ADM and therefore, tumor initiation in the pancreas.

To test if our murine model system is relevant to humans, we established primary acinar cell culture from human pancreas and performed functional ADM assays with various PI3K-PDK1-AKT pathway inhibitors (Figures 7F, 7G, S5A, and S5B). As shown in Figure 7F, TGF-α treatment of human acinar cells induced ADM, as indicated by CK19 staining, with concomitant PI3K pathway activation, as evidenced by AKT-T308 phosphorylation. Treatment with GDC 0941 blocked ADM significantly in a dose-dependent manner (Figures 7G and S5A–S5C) and inhibited AKT-T308 phosphorylation and CK19 expression (Figure S5C).

Overall, these data support the notion that PI3K-PDK1 signaling is essential for pancreatic cell plasticity and tumor initiation and important to transmit the oncogenic Kras-induced program in the pancreas. However, we cannot completely exclude a role for indirect mechanisms of PI3K activation via alternate effectors such as receptor tyrosine kinases, rather than a direct Kras/PI3K interaction (Ardito et al., 2012; Ebi et al., 2011; Navas et al., 2012).
Figure 7. PI3K Signaling Regulates Human and Murine ADM

(A) Genetic strategy used to study the role of PDK1 in KrasG12D-driven ADM.

(B) Quantification of ADM in 6-month-old Ptf1aCre+;LSL-KrasG12D+/+ and Ptf1aCre+;LSL-KrasG12D+/+;Pdk1f/f mice (n = 3; 3 representative slides per mouse).

(C) Phase contrast images of pancreatic acinar cells with the indicated genotypes 5 days after isolation and treatment with or without TGF-α (50 ng/ml).

(D) Quantification of ductal and acinar structures after 5 days in culture. Bar graph shows percentage of structures of the indicated genotypes with and without TGF-α treatment.

(E) Quantification of ductal and acinar structures after 5 days in culture with and without treatment with the indicated chemicals. Bar graph shows percentage of structures of indicated genotype treated with TGF-α and the indicated chemicals.

(F) Confocal microscopic images of TGF-α-induced human ADM. Upper panel: CK19 expression (red) in ADM after 5 days of TGF-α (50 ng/ml) treatment. Lower panel: CK19 expression (red) and AKT-T308 phosphorylation (green) after 5 days TGF-α treatment. Arrow indicates a cell with acinar morphology but positive staining for CK19 and pAKT-T308. Nuclei were counterstained with TO-PRO-3 (blue).

(G) Upper panel: Phase contrast images of human pancreatic acinar cells 5 days after isolation and treatment with TGF-α (50 ng/ml) with or without GDC 0941 (1 μM). Lower panel: Quantification of human ductal and acinar structures from two independent patients after 5 days in culture with and without GDC 0941 treatment. Scale bars, 50 μm. Error bars, ± SEM.

See also Figure S5.
The PI3K/PDK1 Pathway Is a Target for Treatment of Murine and Human Pancreatic Cancer

To test whether PI3K-PDK1-AKT signaling could be a target for pancreatic cancer therapy, we used the well-established Ptf1aCre\(^+\);LSL-Kras\(^{G12D}\);Trp53\(^{R172H}\) (KPC) model (Figure 8A) (Olive et al., 2009). KPC mice develop primary PDAC that faithfully recapitulates the molecular, histopathologic, and clinical features of the human disease (Olive et al., 2009).

To inhibit PI3K signaling in vivo, we used GDC 0941, a potent and selective oral pan class I PI3K inhibitor currently under clinical development (LoRusso et al., 2011; Yuan et al., 2013). GDC 0941 efficiently inhibited the growth of primary murine Kras\(^{G12D}\) and primary human patient-derived PDAC cells in vitro (Figures...
KrasG12D-driven PDAC model had no significant inhibitory effect (Karreth et al., 2011). However, ablation of BrafV600E on KrasG12D driver line, they activated a latent Raf-MEK-ERK pathway at the level of Braf activation or deletion of different Kras-dependent signaling pathways can induce or block PanIN formation. This argues either for the existence of several distinct routes toward PanIN formation, or an essential crosstalk between the Raf-MEK-ERK and PI3K-PDK1-AKT pathway, which might depend on alternate effectors like receptor tyrosine kinases (Adrianto et al., 2012; Ebi et al., 2011; Navas et al., 2012). Collisson and colleagues showed that PanIN formation can be caused by activation of the canonical Raf-MEK-ERK pathway at the level of Braf. Whether Braf is essential for PanIN formation in the KrasG12D model, as we show for the direct PI3K downstream target PDK1, needs to be investigated at the genetic level in the future.

In contrast to our study, Collisson and colleagues found no PanIN development after tamoxifen induced p110αH1047R expression in the pancreas using the Pdx1-CreERT2 mouse line. This might be due to distinct target cells, or differences in Pik3ca copy number, Cre-induced recombination efficacy, or different expression and signaling levels of p110α in the Ptf1aCre and Ela1-CreER mouse and Pdx1-CreERT2 models (Collisson et al., 2012). Based on the similar phenotypes of our KrasG12D and Pik3CAH1047R models, the lack of Ras activation in Pik3CAH1047R mice, comparable levels of PI3K activation and expression as well as PIP3 levels in the KrasG12D and Pik3CAH1047R models, and by showing that deletion of the PI3K effector Pdk1 blocks tumor formation, ADM, and PI3K activation in the KrasG12D model, we provide evidence at multiple levels that Kras acts through PI3K-PDK1 to induce pancreatic cancer. This conclusion is further supported using primary human material showing that PI3K signaling is active in human acinar-to-ductal metaplasia, premalignant pancreatic lesions and cancers, and is mechanistically involved in early processes of pancreatic cell plasticity and cancer formation as well as in tumor maintenance.

Downward and colleagues showed previously that the Ras-PI3K interaction plays an important role in Ras-induced skin and lung carcinogenesis (Gupta et al., 2007). They found that disruption of the direct Ras/p110α interaction—by constitutive expression of Pik3caT208D/K227A in the mouse germline—dramatically reduced the number of Ras-induced papillomas and lung adenomas (Gupta et al., 2007). It is however unclear to what extent this was mediated by cell autonomous or non-cell autonomous effects in the host. Since p110α is required for angiogenesis (Graupera et al., 2008), disruption of the Ras-PI3K interaction in the vasculature, immune system, or stroma is likely to contribute significantly to the observed effects in this model.

Because PDK1 is a central node of PI3K signaling (Castellano and Downward, 2011), we evaluated its cell-autonomous role in Kras-induced NSCLC in vivo. Intriguingly, ablation of Pdk1 specifically in the epithelial compartment of the lung using two different recombination strategies had no significant inhibitory effect on KrasG12D-induced NSCLC development and progression. This suggests that PI3K signaling plays an important role in the tumor microenvironment rather than a cell autonomous function during NSCLC formation (Graupera et al., 2008). This is supported by the observation that Ras-induced NSCLC development is impaired but not completely blocked by constitutive expression of the Pik3caT208D/K227A in the mouse germline (Gupta et al., 2007). In contrast to the lung, genetic inactivation of PDK1 in pancreas epithelium using two different Cre-driver lines completely blocked KrasG12D-induced neoplastic transformation. These
data clearly demonstrate that each tissue has its own and unique cell autonomous signaling requirements during oncogenic transformation. This advance in our understanding of tissue-specific effects of oncogenic Kras may change clinical practice in the future because it clearly shows that results cannot simply be extrapolated from one Kras-driven tumor entity to another.

We have defined the tumor cell autonomous Kras-Pi3K-PDK1 axis as an essential pathway of pancreatic cancer with the capacity to induce cell plasticity, ADAM, PanIN, and cancer formation as well as tumor maintenance. A large variety of pharmacologic inhibitors directed against the Pi3K pathway and the promising targetable node PDK1 are now available for clinical investigation (Engelman, 2009; Liu et al., 2009; Pearce et al., 2010). Recognition of the importance of the Pi3K-PDK1 pathway in PDAC, as demonstrated in this study, will provide a more effective approach for targeted therapeutic interventions for this grave disease.

EXPERIMENTAL PROCEDURES

Mouse Strains and Tumor Models

LSL-KrasG12D/+ (Hingorani et al., 2003), Ptf1aCre/+ (Eser et al., 2011; Nakhai et al., 2007; Seidler et al., 2008), LSL-Rac1G12V/+ (Srinivasan et al., 2009), LSL-Trp53R172H/+ (Hingorani et al., 2005), Trp53f/f (Jonkers et al., 2001), LSL-R26Rtm (Seidler et al., 2008), R26mT/mG (Muzumdar et al., 2007), EIA1-CreERTM (Stanger et al., 2005), Cdkn2a−/− (Agirre et al., 2003), Craf−/− (Jesenberger et al., 2001), and Pdk1f/f mice have been described previously. The strains were interbred to obtain mice with activation of distinct pathways in the pancreas as previously described (Eser et al., 2011). All animal studies were conducted in compliance with European guidelines for the care and use of laboratory animals and were approved by the Institutional Animal Care and Use Committees (IACUC) of Technische Universität München, Regierung von Oberbayern and UK Home Office.

Human Pancreatic Tissue Samples

This study was conformed to the Declaration of Helsinki and was approved by the ethics committee of the Technische Universität München. Informed consent was obtained from all patients included in the study.

Generation of Primary Human and Murine Ductal Pancreatic Cancer Cell Lines

Primary dispersed murine pancreatic cancer cells were established from Ptf1aCre/+;LSL-KrasG12D/+ and Ptf1aCre/+;LSL-Pik3caH1047R/+ mice and cultured as previously described (von Burstin et al., 2009). Primary dispersed human PDAC cells were isolated from surgically resected human PDAC as recently described (Conradt et al., 2011). Only early-passage (passage 3 to 4) dispersed cells were used for assays.

Establishment of Patient-Derived Xenograft Tumors from Primary Human PDAC

Tissues were placed into chilled sterile RPMI 1640 (Thermo Scientific/Hyclone, Waltham, MA) supplemented with 1% (v/v) penicillin/streptomycin/ampicillin B. The tumor was washed twice, dissected into 3 mm cubes and pieces were implanted subcutaneously into NOD SCID IL2Rγ− mice (#005557; Jackson Laboratory, Bar Harbor, Maine) within 1 hour of resection.

Patient-Derived Orthotopic Human Pancreatic Cancer Xenotransplantation Model

One million patient-derived primary human pancreatic cancer cells in 20 μl Dulbecco’s modified Eagle medium were implanted orthotopically into the pancreas of NSG mice as described (von Burstin et al., 2009; von Burstin et al., 2008).

Statistical Analyses

Comparisons between data sets were made with analysis of variance, followed by Student’s t test. A Bonferroni correction of the p values was performed for multiple testing. Kaplan-Meier survival curves were compared by log-rank test. Values of p < 0.05 or less were considered to be statistically significant.

SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.ccr.2013.01.023.

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