ABSTRACT

KRAS mutation is a hallmark of pancreatic ductal adenocarcinoma (PDA) but remains an intractable pharmacologic target. Consequently, defining RAS effector pathway(s) required for PDA initiation and maintenance is critical to improve treatment of this disease. Here, we show that expression of BRAF\(^{V600E}\), but not PIK3CA\(^{H1047R}\), in the mouse pancreas leads to pancreatic intraepithelial neoplasia (PanIN) lesions. Moreover, concomitant expression of BRAF\(^{V600E}\) and TP53\(^{R270H}\) result in lethal PDA. We tested pharmacologic inhibitors of RAS effectors against multiple human PDA cell lines. Mitogen-activated protein (MAP)/extracellular signal–regulated (ERK) kinase (MEK) inhibition was highly effective both in vivo and in vitro and was synergistic with AKT inhibition in most cell lines tested. We show that RAF→MEK→ERK signaling is central to the initiation and maintenance of PDA and to rational combination strategies in this disease. These results emphasize the value of leveraging multiple complementary experimental systems to prioritize pathways for effective intervention strategies in PDA.

SIGNIFICANCE: PDA is difficult to treat, in large part, due to recurrent mutations in the KRAS gene. Here, we define rational treatment approaches for the disease achievable today with existing drug combinations through thorough genetic and pharmacologic dissection of the major KRAS effector pathways, RAF→MEK→ERK and phosphoinositide 3′-kinase (PI3′K)→AKT. Cancer Discov; 2(8): 685–93. ©2012 AACR.

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Note: Supplementary data for this article are available at Cancer Discovery Online (http://cancerdiscovery.aacrjournals.org/).

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doi: 10.1158/2159-8290.CD-11-0347
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INTRODUCTION

Pancreatic ductal adenocarcinoma (PDA) poses a major challenge in oncology due to our inability to diagnose the disease early in its progression, its aggressive clinical behavior, and the lack of effective systemic chemotherapy (1). The vast majority of PDAs harbor a mutationally activated form of KRAS (2). Moreover, KRAS mutation is an early event in PDA, as evidenced by its high prevalence in pancreatic intraepithelial neoplasia (PanIN) lesions, thought to be a benign precursor to malignant PDA (3). Furthermore, widespread expression of KRASG12D throughout the developing mouse pancreas leads to multifocal PanIN formation and, when combined with lesions in p53, to PDA with high frequency in adult mice (4, 5).

Mutational activation of KRAS binds to a multiplicity of effector proteins including RAF kinases, phosphoinositide 3′-kinase (PI3K), and guanine nucleotide exchange factors for RAL and RHO GTPases, respectively (6). Because mutationally activated RAS remains an intractable pharmacologic target, defining relevant RAS effector pathway(s) in PDA is of considerable clinical importance. Because potent and specific inhibitors of key components of RAS effector pathways are being clinically deployed in a number of malignancies, it has become crucial to understand how best to implement these drugs in the clinical arena for maximal efficacy while minimizing toxicity. Unlike the scenario in melanoma or colorectal cancer, mutational activation of RAS effectors (e.g., BRAF or PIK3CA) is extremely rare in PDA and therefore uninformative as to the key downstream mediators of RAS signaling (7). This might suggest that numerous RAS effector pathways are essential for PDA and that effective targeting of cancers maintained by mutationally activated RAS remains an intractable pharmacologic challenge in oncology due to our inability to diagnose the disease early in its progression, its aggressive clinical behavior, and the lack of effective systemic chemotherapy (1). The vast majority of PDAs harbor a mutationally activated form of KRAS (2). Moreover, KRAS mutation is an early event in PDA, as evidenced by its high prevalence in pancreatic intraepithelial neoplasia (PanIN) lesions, thought to be a benign precursor to malignant PDA (3). Furthermore, widespread expression of KRASG12D throughout the developing mouse pancreas leads to multifocal PanIN formation and, when combined with lesions in p53, to PDA with high frequency in adult mice (4, 5).

Mutational activation of KRAS binds to a multiplicity of effector proteins including RAF kinases, phosphoinositide 3′-kinase (PI3K), and guanine nucleotide exchange factors for RAL and RHO GTPases, respectively (6). Because mutationally activated RAS remains an intractable pharmacologic target, defining relevant RAS effector pathway(s) in PDA is of considerable clinical importance. Because potent and specific inhibitors of key components of RAS effector pathways are being clinically deployed in a number of malignancies, it has become crucial to understand how best to implement these drugs in the clinical arena for maximal efficacy while minimizing toxicity. Unlike the scenario in melanoma or colorectal cancer, mutational activation of RAS effectors (e.g., BRAF or PIK3CA) is extremely rare in PDA and therefore uninformative as to the key downstream mediators of RAS signaling (7). This might suggest that numerous RAS effector pathways are essential for PDA and that effective targeting of cancers maintained by mutationally activated RAS might require concomitant inhibition of 2 or more RAS effector pathways (8).

We examined the requirements of the RAF or PI3K effector arms of KRAS signaling in the initiation, progression, and maintenance of PDA using genetically engineered mouse (GEM) cancer models and cancer cell lines derived from human or mouse PDA. Whereas pancreas-specific expression of BRAFV600E led to the rapid formation of multifocal PanIN lesions, similarly initiated expression of PIK3CAH1047R was without obvious effect. Furthermore, combined expression of BRAFV600E and gain-of-function TP53R270H uniformly led to lethal PDA in the mouse. Oral delivery of a mitogen-activated protein (MAP)/extracellular signal–regulated (ERK) kinase (MEK) inhibitor was effective in inhibiting ERK phosphorylation in vivo in an established, autochthonous model of PDA reported to exclude drugs and prolonged survival in a novel syngeneic model of PDA. Pharmacologic inhibition of MEK potently suppressed proliferation in a subset of PDA-derived cell lines in vitro but induced activation of AKT in both KRAS wild-type (w/t) or mutated human PDA cell lines. Finally, combined MEK and AKT inhibition showed synergistic interactions in most human PDA cells tested. Overall, our findings suggest the potential use of concerted clinical efforts to completely inhibit the RAS→RAF→MEK→ERK pathway at or below MEK in a subset of patients with PDA and to develop tolerable combination regimens of MEK and AKT inhibitors in this disease.

RESULTS

Expression of BRAFV600E But Not PIK3CAH1047R Is Sufficient for PanIN Formation

To test the consequences of activating the RAF→MEK→ERK pathway specifically in the pancreas, we crossed p48Cre mice with BrafCA mice. As described previously, BrafCA encodes normal BRAF but following Cre-mediated recombination is rearranged to encode activated BRAFV600E (9). p48Cre expresses Cre recombinase in place of the Pitf gene. No compound p48Cre; BrafCA progeny were detected at the time of weaning, leading us to conclude that widespread expression of BRAFV600E in the developing mouse pancreas is incompatible with development to adulthood. This lethality contrasts with the viability of p48Cre; KrasG12D mice (10). To circumvent this lethality, we generated compound Pdx1::CreERT2; BrafCA mice (BC mice hereafter) where expression of BRAFV600E is induced in the adult pancreas under the control of a conditionally active Cre recombinase driven by the Pdx1 promoter (11). BC mice were born at normal Mendelian ratios and were healthy and fertile. In parallel, and as a comparator, we generated a cohort of Pdx1::CreERT2; KrasG12D mice (KC mice). Cohorts of BC and KC mice were treated with tamoxifen at P14 to initiate Cre activity and thereby BRAFV600E or KRASG12D expression in the pancreas. Mice were euthanized for analysis around P100, and all mice were healthy at the time of euthanasia.

Pancreatic expression of BRAFV600E led to near total replacement of the exocrine pancreas with PanIN lesions (Fig. 1A and B). These lesions were morphologically indistinguishable from those arising in KC mice and were of similar grade although greater in number (Fig. 1C and not shown). PanINs from BC mice expressed the ductal marker cytokeratin (CK) 19 (Fig. 1D), Ki67 (a marker of proliferation; Fig. 1E), and had abundant phosphorylated nuclear ERK1/2 (Fig. 1F), indicating activation of the RAF→MEK→ERK pathway. In addition, whereas primary cilia were observed in both pancreatic islets and normal ducts, PanIN cells from BC mice lacked primary cilia (Fig. 1G and H), consistent with previous findings in KRASG12D-induced induced PanIN lesions (12). Six BC mice aged 1 year showed no evidence of PDA upon euthanasia.

To test the ability of activated PI3K-α to initiate PanIN formation, we generated Pdx1::CreERT2; Pik3caAla1047R (PC) mice. The Pik3caAla1047R allele encodes normal PI3K-α before Cre-mediated recombination after which mutational activation PI3K-α (PIK3CAH1047R) is expressed from the endogenous Pik3ca locus (13). We used a specific PCR to show that recombination (and thus activation) of the Pik3caAla1047R allele in the pancreas occurred (not shown) but found neither detectable PanIN lesions nor any other pancreatic abnormalities in PC mice up to 6 months after Cre induction with tamoxifen. These data indicate that mutationally activated BRAFV600E, but not PIK3CAH1047R, can initiate PanIN formation with an efficiency equal to that of KRASG12D.

BRAFV600E Cooperates with Gain-of-Function TP53R270H for PDA Formation

Mutational activation KRASG12D cooperates with gain-of-function TP53R270H to promote development of PDA with...
high penetrance and striking histologic and clinical similarity to the human disease (5). We generated a cohort of Pdx1::CreER\textsuperscript{T2}, Braf\textsuperscript{CA/+}, Tp53\textsuperscript{LSL-R270H} mice (BPC mice hereafter) to test whether oncogenic BRAF\textsuperscript{V600E} might display similar cooperation. All BPC mice required euthanasia at 4.5 to 9 months because of abdominal distention, wasting and substantial loss of body weight. Mice typically presented with ascites and extra-pancreatic spread of metastatic disease, most often to the liver, peritoneal cavity, and lung (Fig. 2A–E). All BPC mice displayed clear evidence of PDA at necropsy. Analysis of tumor-derived genomic DNA confirmed recombination of the Braf\textsuperscript{CA} allele and excluded spurious acquisition of activating mutations in either exon 1 of Kras or exon 20 of Pik3ca (data not shown). Histologic examination of these cancers showed them to be moderately differentiated PDA displaying robust proliferation (Ki67; Fig. 2F), heterogeneous CK19 expression (Fig. 2G), and abundant phosphorylated ERK (pERK; Fig. 2H). All mice succumbed to disease within a year (Fig. 2I). Interestingly, both BRAF\textsuperscript{V600E}-induced PanINs and PDAs displayed abundant stroma and a desmoplasia similar to that seen within the human disease. We concluded that oncogenic BRAF\textsuperscript{V600E} substitutes for most, if not all, of the oncogenic functions of KRAS\textsuperscript{G12D} in the initiation and progression of PDA in the mouse.

**Bioavailability of MEK1/2 Inhibitor PD325901 in PDA**

The above results suggested to us that although mutant Kras can serve many oncogenic functions in cancer, activation of the RAF pathway alone satisfied a genetic sufficiency argument for the PanIN and PDA initiation. To translate this finding into a more clinically relevant hypothesis, we next considered the clinical use of MEK inhibitors and potential barriers to successful trials with these agents in PDA. It has been recently reported that chemotherapeutic agents are excluded from PDA tissue due to poor drug perfusion into the primary tumor, which is in turn attributable to poor tumor vascularization (14, 15). To interrogate whether MEK1/2 inhibitor PD325901 was bioavailable to PDA tissue, we used an autochthonous Kras\textsuperscript{LSL-G12D}, Tp53\textsuperscript{LSL-R270H}, p48\textsuperscript{Cre} (KPC) mouse model similar to that previously described to exhibit high interstitial pressures and to exclude various...
Figure 2. BRAF\textsuperscript{V600E} and TP53\textsuperscript{R270H} cooperate to form lethal PDA resembling the human disease. A, a 6-month-old Pdx1::CreER\textsuperscript{T2}; Braf\textsuperscript{CA/+}; Trp53\textsuperscript{LSL-R270H} (BPC) mouse with ascites. B, gross images of primary pancreatic tumor (black arrow) and omental metastases (blue arrows). C, hematoxylin and eosin (H&E) staining of primary PDA arising in the pancreas of a BPC mouse. H&E staining of (D) liver metastases (black arrows) or (E) lung metastases (black arrow) from same. PDAs arising in BPC mice are proliferative (Ki67; F), heterogeneously express ductal markers (CK19; G), and display high levels of MAPK activation (pERK; H). I, survival curves of mice of indicated genotypes.
drugs from PDA tissues (14, 15). In this model, 2 daily treatments with MEK1/2 inhibitor PD325901 led to a profound reduction in pERK as detected by immunohistochemistry (Fig. 3A–D), suggesting that this agent is bioavailable to PDA cells in vivo at clinically achievable doses. We concluded that sufficient levels of MEK1/2 inhibition might be pharmacologically feasible in PDA, despite the drug delivery challenges posed by hypovascularity and desmoplasia in this disease.

For drug efficacy studies, we next developed 2 new in vivo, mouse syngeneic orthotopic models of PDA, denoted INK4.1<sup>−/−</sup>Luc<sup>+</sup> and p53−/− <sup>−/−</sup>Luc<sup>+</sup>, using previously described mouse PDA-derived cell lines engineered to express luciferase (16). We found that implantation of either line in the pancreas of immune competent FVB/n mice reproducibly led to PDA with characteristics of the clinical disease including recruitment of activated stroma (Supplementary Fig. S1), ascites, cachexia, and bowel obstruction requiring euthanasia at 5 to 6 weeks postimplantation. The predictable kinetics and quantifiable tumor implantation allowed for relatively economical drug efficacy studies, as compared with the
autochthonous model (17). Following orthotopic engraftment, tumors-bearing mice were divided into equal tumor-bearing groups (as quantified by bioluminescence), treated with either vehicle or MEK1/2 inhibitor PD325901 by gavage for 14 days, and monitored clinically daily for disease progression. PD325901 led to pERK reduction 6 hours after a single oral gavage (Supplementary Fig. S2A and S2B), indicating that the drug gains access to tumor cells in this model as in the autochthonous model. Treated mice were healthy while receiving drug, whereas control-treated mice began to decline. The experiment was terminated when the final vehicle-treated mouse required euthanasia, as dictated by Institutional Animal Care and Use Committee (IACUC) protocols at our center. By this analysis, MEK inhibition resulted in a statistically significant survival advantage in mice bearing either INK4.1<sup>avr,Luc</sup> (log-rank, \( P = 0.043 \)) or p53 2.1<sup>avr,Luc</sup> (log-rank, \( P < 0.01 \)) syngeneic orthotopic xenografts. Despite this survival advantage, we noted that MEK inhibition was mostly cytostatic, as noted in vitro (18), and upon cessation of PD325901, all treated mice displayed rapid tumor growth (Fig. 3E–H).

**RAS Pathway Dependencies of Human PDA Cell Lines**

We next sought to complement our analysis of GEM models of PDA by expanding a previously reported panel of human PDA cell lines with the goal of better representing the heterogeneity of PDA (Supplementary Table S1; ref. 16). To pharmacologically assess the dependence of human PDA cells on specific signaling nodes downstream of RAS, we exposed all cell lines to MEK1/2 inhibitors (GSK1120212 or PD325901), RAF inhibitor (GDC-0879), or AKT1/2 inhibitor (GSK690693). In addition, cell lines were treated with pairwise combinations of these agents to probe for synergistic growth inhibition by targeting both RAF→MEK→ERK and PI3K→AKT signaling simultaneously in the same cells.

RAF inhibitor GDC-0879 led to only minor inhibitory effects on PDA cell proliferation when used alone. Furthermore, RAF inhibition clearly antagonized the antiproliferative effects of MEK1/2 inhibition when RAF inhibitor GDC-0879 was co-administered with MEK1/2 inhibitor PD325901 (Supplementary Fig. S3A). Western blotting for pERK confirmed that RAF inhibitor GDC-0879 indeed augmented RAF→MEK→ERK signaling in KRAS-mutant Sui2 cells, essentially antagonizing MEK1/2 inhibition (Supplementary Fig. S3B). Consequently, neither GDC-0879 nor its combinations were pursued further.

To examine the effects of MEK and AKT inhibition while maximizing translational relevance, we used a MEK inhibitor currently in clinical trials with other agents, GSK1120212 [e.g., NCT00955773 and NCT01138085 (19)]. IC<sub>50</sub> values for the MEK inhibitor GSK1120212 were dynamic across the cell line panel (Fig. 4A). We found that MEK inhibition led to induction of pAKT (a marker of activation of PI3K→AKT signaling in several PDA lines (Supplementary Fig. S4). Consistent with this effect, we found that while single-agent AKT inhibition with GSK690693 had minimal effects on its own, combination treatment with GSK1120212 and GSK690693 led to statistically significant synergy in most PDA lines tested (Fig. 4B and C; Supplementary Fig. S5 and Supplementary Table S2; ref. 20). Treatment with GSK1120212 led to a decrease in phosphorylation of ERK, pS6, and 4EBP1 (Thr37/46). GSK690693 treatment led to an expected increase in AKT phosphorylation and suppression of phosphorylation of the direct AKT target PRAS40 (pT246). The combination of the 2 inhibitors had a more profound inhibitory effect on both RPS6 and 4EBP1 phosphorylation in most of the PDA cell lines than single-agent treatments (Fig. 4D). We concluded that while some PDA lines are sensitive to MEK1/2 inhibition alone, the addition of AKT inhibition consistently potentiated responses, as evaluated by formal drug synergy analysis.

**DISCUSSION**

The strikingly poor prognosis of patients with PDA is largely attributable to late diagnosis and general resistance to conventional cytotoxic or targeted therapeutics. Although mutational activation of KRAS is a signature genetic event of PDA, approaches to directly inhibit constitutively active, GTP-bound RAS proteins have so far failed. Consequently, considerable attention has shifted to pharmacologically tractable targets acting downstream of RAS-GTP on the arms of its various effector pathways. Chief among these are the RAF→MEK→ERK and the PI3K→AKT pathways for 2 reasons. First, the RAF and PI3K kinases are themselves frequently mutationally activated in human cancer whereas other putative RAS effectors are not. Second, components of these pathways are targeted with agents in clinical development. In this study, we sought to explore the relative importance of these RAS effector pathways in PDA initiation and maintenance to better prioritize treatment approaches with such pathway inhibitors and to prospectively define combinations of inhibitors likely to be of benefit specifically in this lethal disease. A key conclusion of this research is that induced expression of BRAF<sup>V600E</sup>, but not PIK3CA<sup>H1047R</sup>, signaling can recapitulate the PDA phenotype endowed by mutant KRAS<sup>G12D</sup> in mice. Moreover, pharmacologic inhibition of MEK has antitumor effects against a subset of PDAs and broadly synergizes with AKT inhibition in this disease. Whereas KRAS mutation is nearly universal in PDA, mutational activation of either BRAF or PIK3CA is very uncommon (7). It is perhaps surprising then that BRAF<sup>V600E</sup> is able to phenocopy the effects of KRAS<sup>G12D</sup> in the mouse with such efficiency. These data suggest that, in the mouse, little more is required of KRAS<sup>G12D</sup> than activation of the RAF→MEK→ERK axis for PDA initiation. In this capacity, activated BRAF (like activated KRAS), appears capable of activating additional pathways (e.g., Myc, NF-κB) and processes (inflammation, stromal recruitment) necessary for pancreatic tumorigenesis. Moreover, the absence of an overt pancreatic phenotype in Pdx1<sup>–/–</sup>CreER; Pkb3<sup>CreLacZ</sup> mice further emphasizes the relative specificity of the RAF→MEK→ERK pathway in PDA initiation. These results are consistent with GEM models of KRAS<sup>G12D</sup>-induced lung tumorigenesis wherein RAF→MEK→ERK signaling is both necessary (21) and sufficient (9, 22) for tumor initiation in lung and agrees with the requirement for RAF in RAS-induced skin cancer (23).

There are numerous inhibitors of RAF→MEK→ERK and PI3K→AKT signaling currently in drug development (6). Our findings support the contraindication of RAF inhibitors in the treatment of cancers driven by mutationally
Figure 4. Combined inhibition of MEK and AKT leads to synergistic effects across a large panel of PDA cell lines. A, IC_{50} measurements of human PDA cell lines treated with MEK inhibitor GSK1120212. Cell lines are on the x-axis and IC_{50} (mol/L) is on the y-axis. Representative dose-response curves of (B) 3.27 or (C) Sw1990 treated with GSK690693 (triangles), GSK1120212 (diamonds), or a 5:1 mol/L fixed-dose combination ratio of GSK1120212:GSK690693 (squares) plotted as the dose of GSK1120212 in the combination. The x-axis is drug concentration in mol/L and y-axis is percentage of growth inhibition at 72 hours. Error bars are ±SD. D, Immunoblotting of a subset of human PDA cell lines treated with dimethyl sulfoxide (DMSO), MEK inhibitor GSK1120212 (212) at 200 nmol/L, AKT inhibitor GSK690693 (69) at 1 μmol/L, or a 0.2:1 μmol/L (212:69) combination of the 2 agents for 24 hours.
activated RAS proteins due to their lack of efficacy and likely growth-stimulatory characteristics (24). We find that MEK1/2 inhibitor has potent antitumor activity against human or mouse PDA cell lines and against orthotopically implanted tumors. We observed mostly cytostatic responses following MEK inhibition in vitro and observed induction of AKT signaling in response to MEK inhibition in PDA cells. This suggested a functional feedback loop as observed by others in breast or colorectal cancer lines harboring RAS mutations (25, 26). Indeed, we found that combined inhibition of MEK and AKT led to synergistic effects in the majority of human PDA cell lines tested, similar to findings in lung cancer (27). We interpret our findings with those of others to suggest that PDA cells, while relatively resistant to single-agent AKT inhibition, appear to consistently recruit this important survival pathway in response to acute MEK inhibition, possibly explaining the synergistic interactions seen with these 2 classes of agents.

In total these findings emphasize the central role played by RAF→MEK→ERK signaling in both the genesis and maintenance of PDA. These results are important because, although KRAS remains a pharmacologically intractable target, there are numerous potent kinase inhibitors being developed against the downstream effectors of RAS. However, regimens to structure the combined use of such inhibitors remain to be established. We show that agents currently in clinical trials show potent synergy in PDA treatment. These findings strongly support the further development of combined MEK and AKT inhibition in PDA and suggest a clear direction for the implementation of pathway-targeted approaches in this disease with tremendous unmet medical need.

METHODS

Mouse Studies

All experiments were approved by the IACUC of the University of California, San Francisco. Brca2−/− (9), KrasLSL-G12D (4), Trp53LSL-R270H (14), Pdx1-CreER(T2) (11), and PknScf(1–208) (13) mice and their genotyping protocols have been described. Tamoxifen was dissolved in peanut oil and delivered in one intraperitoneal injection on day 14 of life. Derivation of the p53 2.1.1 and INK4.1 lines from FVB/n mice has been described (16). These cells were transduced in vitro with a lentiviral vector encoding a firefly luciferase and mCherry (pLV406G) and selected in G418 to give rise to INK4.1 syn_Luc and p53 syn_Luc (8). The INK4.1 syn_Luc line was used in all experiments.

Human Studies

Sensitivity Testing

PD325901 was obtained from Hunsan International Trading; GDC0879 was provided by Genentech, and GSK690693 were from ChemieTek. All were dissolved in dimethyl sulfoxide. PDA cell lines were plated on day 0, treated with nine, 2-fold dilutions of single drug or 1:1 mol/L (PD325901:GDC0879) or 5:1 mol/L (GSK690693:GSK1120121) ratio combinations of drugs, and read as described (16). IC50 and synergy assessments were conducted using the SYNERGY program in R (see Supplementary Methods).

Immunoblotting

Cells were grown to 70% confluence and then either treated with 1 μmol/L GSK690693 or 200 nmol/L GSK1120121 for 24 hours. Cells were lysed using radioimmunoprecipitation assay buffer and loaded onto NuPAGE Bis-Tris SDS gels. Antisera included anti-phospho-ERK1/2, anti-ERK1, anti-phospho-AKT (pS473), anti-pan-Akt, anti-phospho-PRAS40, anti-phospho-S6RP and phospho-4EBP1 from Cell Signaling Technology, and anti-β-actin was obtained from Santa Cruz Biotechnology. The LiCOR Odyssey system was used to visualize immunoblots.

Immunohistochemistry and Immunofluorescence

Pancreata were fixed overnight in formalin, embedded in paraffin, cut into 5-μm sections, and placed on slides. Following citrate-mediated antigen retrieval, slides were incubated with monoclonal rabbit antibodies against Ki67 (Abcam), pERK1/2 (Cell Signaling Technology), rat anti-CK19 (Hybridoma Bank at the University of Iowa, Iowa City, IA), p53 sc6243 (Santa Cruz), and anti-smooth muscle actin A2547 (Sigma) overnight followed by incubation with biotinylated goat anti-rabbit or goat anti-rat IgG and horseradish peroxidase (Vector). Detection was conducted using the DAB Chromogen System (Dako). Primary cilia were stained and detected as described (12).

Disclosure of Potential Conflicts of Interest

J.W. Gray has commercial research grants from GlaxoSmithKline, Pfizer, and Susan G. Komen for the Cure and is a consultant/advisory board member for New Leaf Ventures, Agenda, and KromaTiD. M. McMahon has a commercial research grant from the National Comprehensive Cancer Network. No potential conflicts of interest were disclosed by the other authors.

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Conception and design: E.A. Collisson, D. Dankort, M. McMahon
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PA-TU8988T, and ASPC-1 were provided by Lynda Chin (MD Anderson, Houston, TX). CFp1a1, MiaPaca2 HPAC, and Panc1 are from American Type Culture Collection. All cell lines were genotyped by Affymetrix SNP N6.0 for definitive future disambiguation of provenance. No additional authentication was conducted by the authors.

Sensitivity Testing

PD325901 was obtained from Hunsan International Trading; GDC0879 was provided by Genentech, and GSK690693 were from ChemieTek. All were dissolved in dimethyl sulfoxide. PDA cell lines were plated on day 0, treated with nine, 2-fold dilutions of single drug or 1:1 mol/L (PD325901:GDC0879) or 5:1 mol/L (GSK690693:GSK1120121) ratio combinations of drugs, and read as described (16). IC50 and synergy assessments were conducted using the SYNERGY program in R (see Supplementary Methods).
RAF→MEK→ERK Pathway in Pancreatic Ductal Adenocarcinoma

Acknowledgments

The authors thank Katie Bell, Anthony Karnezis, Grace Kim, Donghui Wang, Don Hom, Paul Phojanakong, Neerine Affara, Jeff Chang, Matthias Hebrok, John P. Morris IV, and Stephan Gysin for assistance, advice, and discussion. We thank Dr. Tyler Jacks and Dr. David Tuveson for access to KrasG12D and Trp53R172H. Douglas Melton for Pdx1:CreERT2, and Christopher Wright for p48Cre mice. The Preclinical Therapeutics Core and Mouse Pathology Cores at the Helen Diller Family Comprehensive Cancer Center are acknowledged.

Grant Support

E.A. Collisson is supported by NIH/NCI CA137153. Funds were provided by the Noren Fund for Pancreatic Cancer Research (to M. McMahon). WA. Phillips is supported by project grants from the National Health and Medical Research Council of Australia. This research was supported by NIH/NCI Grants P50 CA 58207, U54 CA 112970, and NHGRI U24 CA 126551; by the Department of the Army, Award W81XWH-07-1-0663, to J.W. Gray. Funds were provided by the Noren and Hasbun Funds for Pancreatic Cancer Research, and NIH/NCI R01 CA121361 to M. McMahon.

Received January 3, 2012; revised May 8, 2012; accepted May 18, 2012; published OnlineFirst May 24, 2012.

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