

Oncogenic KRAS Activates Hedgehog Signaling Pathway in Pancreatic Cancer Cells^{*[5]}

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Hedgehog (Hh) signaling is deregulated in multiple human cancers including pancreatic ductal adenocarcinoma (PDA). Because KRAS mutation represents one of the earliest genetic alterations and occurs almost universally in PDA, we hypothesized that oncogenic KRAS promotes pancreatic tumorigenesis in part through activation of the Hh pathway. Here, we report that oncogenic KRAS activates hedgehog signaling in PDA cells, utilizing a downstream effector pathway mediated by RAF/MEK/MAPK but not phosphatidylinositol 3-kinase (PI3K)/AKT. Oncogenic KRAS transformation of human pancreatic ductal epithelial cells increases GLI transcriptional activity, an effect that is inhibited by the MEK-specific inhibitors U0126 and PD98059, but not by the PI3K-specific inhibitor wortmannin. Inactivation of KRAS activity by a small interfering RNA specific for oncogenic KRAS inhibits GLI activity and GLI1 expression in PDA cell lines with activating KRAS mutation; the MEK inhibitors U0126 and PD98059 elicit a similar response. In addition, expression of the constitutively active form of BRAF^{E600}, but not myr-AKT, blocks the inhibitory effects of KRAS knockdown on Hh signaling. Finally, suppressing GLI activity leads to a selective attenuation of the oncogenic transformation activity of mutant KRAS-expressing PDA cells. These results demonstrate that oncogenic KRAS, through RAF/MEK/MAPK signaling, is directly involved in the activation of the hedgehog pathway in PDA cells and that collaboration between these two signaling pathways may play an important role in PDA progression.

Pancreatic ductal adenocarcinoma (PDA)² is the fourth leading cause of cancer-related death for both men and women in the United States. It was estimated that in 2006 33,730 new

cases would be diagnosed, and 32,300 would die from the disease (*American Cancer Society Cancer Facts and Figures 2006*). Therefore, PDA is one of the most lethal human diseases, with a 5-year survival rate of less than 4% and a median survival of less than 6 months.

PDA is one of the better-characterized neoplasms at the genetic level. There are now sufficient clinical, genetic, and pathological data to support a tumor progression model for PDA in which the pancreatic ductal epithelium progresses from normal to increased grades of pancreatic intraepithelial neoplasia to invasive cancer (1, 2). Accompanying the progressive morphological changes is the sequential accumulation of genetic alterations in the KRAS oncogene and the tumor suppressors INK4A, p53, and SMAD4/DPC4, although these alterations have not been linked to the acquisition of specific histopathological attributes (3–5). In addition to these frequent genetic abnormalities, mutations in the tumor suppressors BRCA2, TGFBR1, and TGFBR2, the serine-threonine kinases AKT2 and LKB1/STK11, and certain DNA mismatch-repair genes represent other less common genetic events in PDA (6–11).

Aberrant RAS activation plays a critical role in tumorigenesis; activating RAS mutations are found in 30% of all human cancers (12). Of all human cancers, PDA has the highest incidence of activating KRAS mutations (13). Activating KRAS mutations, representing the earliest genetic changes associated with the transformation of normal ductal epithelium and PDA development, have been detected in pancreatic duct lesions with minimal cytological and architectural atypia and occasionally in histologically normal pancreas (3, 14–17). The frequency of KRAS mutations correlates with disease progression, reaching almost 100% in pancreatic adenocarcinomas. Targeted endogenous expression of an oncogenic KRAS allele in the mouse pancreas is sufficient to drive the development of pancreatic intraepithelial neoplasia and subsequently at low frequency the progression to both locally invasive adenocarcinoma and metastatic disease with sites of spread exactly as found in human pancreatic cancer (18–20). These observations suggest that KRAS plays an essential role in the initiation, development, and maintenance of PDA.

Recently, the hedgehog (Hh) signaling pathway has been implicated as playing an important role in the progression and maintenance of PDA (21–23). Hh signaling is essential for morphogenesis, tissue patterning, and stem cell maintenance in metazoan embryos (24). Hh binds to its membrane receptor Patched (PTC), releasing PTC inhibition of a seven-transmembrane protein, Smoothened (SMO), which in turn activates

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² The abbreviations used are: PDA, pancreatic ductal adenocarcinoma; FTS, S-trans,transfarnesylthiosalicylic acid; GLI, glioblastoma gene product; Hh, hedgehog; HPDE, human pancreatic ductal epithelium; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; PI3K, phosphatidylinositol 3-kinase; Shh, Sonic hedgehog; SMO, smoothened gene product; siRNA, small interfering RNA.

downstream cytoplasmic transcription factors; that is, the CI protein in *Drosophila* or the mammalian homologue GLI proteins (25). Components of the Hh signaling pathway, including the ligand and the receptors, are overexpressed in human PDA tissues and cell lines. Suppressing Hh activity using cyclopamine, a steroidal alkaloid that inhibits Hh signaling through direct interaction with SMO (26), in some PDA cells with activated Hh signaling, can inhibit cell growth *in vitro* and reduce tumor growth *in vivo* in the xenograft and orthotopic mouse model (21–23, 27).

The coincidence of uncontrolled activation of the RAS and Hh pathways in the early stages of PDA suggests that cross-talk between these two pathways may be a very important mechanism for the initiation and development of PDA. However, the causal effects between KRAS and Hh signaling in pancreatic tumorigenesis are not clear. Earlier results from *Pdx-Shh* mice had suggested that ectopic expression of Hedgehog ligands is sufficient to activate the Ras signaling pathway by inducing a mutation in the *Kras* gene (21), and a recent study indicates that cell-autonomous activation of the Hedgehog pathway is not sufficient to induce mutations in the *Kras* gene or to activate MAPK downstream of Ras (28). In addition, although expression of endogenous level of oncogenic *Kras*, *Kras*^{G12D}, leads to pancreatic intraepithelial neoplasia identical to all three stages found in the cognate human condition and eventually PDA in mice (19), activation of Hh signaling alone is not sufficient to induce pancreatic intraepithelial neoplasia and PDA in a mouse model in which Hh signaling is activated specifically in the pancreatic epithelium (28). Because KRAS mutation represents one of the earliest genetic alterations and occurs almost universally in pancreatic adenocarcinomas, we hypothesized that oncogenic KRAS promotes pancreatic tumorigenesis in part through activation of the Hh signaling pathway in PDA. Our study shows that oncogenic transformation of human pancreatic ductal epithelial (HPDE) cells by oncogenic KRAS is indeed accompanied by enhanced GLI activation and that specific down-regulation of oncogenic KRAS activity inhibits Hh signaling in PDA cell lines with KRAS mutations. These results demonstrate that oncogenic KRAS is involved in activation of the Hh/GLI pathway in PDA cells and that cross-talk between the oncogenic KRAS and Hh pathways may play an important role in promoting cancer development during pancreatic tumorigenesis.

EXPERIMENTAL PROCEDURES

Reagents—Wortmannin, PD98059, U0126, and MG132 were purchased from Calbiochem. *S*-Trans,transfarnesylthiosalicylic acid (FTS) was provided by Dr. Victor J. Bauer (Concordia Pharmaceuticals). Cyclopamine and tomatidine were purchased from Toronto Research Chemicals (North York, Ontario, Canada). Cycloheximide was from Sigma. Mouse anti-RAS antibodies, rabbit anti-MAPK, anti-phospho-MAPK, anti-AKT, anti-phospho-AKT, anti-phospho-glycogen synthase kinase 3 β , and anti-Myc antibodies were obtained from Cell Signaling Inc. (Beverly, MA). Mouse anti-KRAS antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse anti- α -tubulin was from Molecular Probe (Eugene, OR). Horseradish peroxidase-

conjugated anti-mouse and anti-rabbit secondary antibodies were from Jackson Immunolabs (West Grove, PA). KRAS^{D12}-specific siRNA duplex and control siRNA were obtained from Dharmacon RNA Technologies (Lafayette, CO). *Gli* luciferase reporter constructs including 8 \times 3' *Gli* BSwT-luc and 8 \times 3' *Gli* BSmT-luc were described previously (29). Plasmids for expression of *Renilla* luciferase (pRL-SV40-luc) were from Promega (Madison, WI). pCS2-MT and pCS2-MT-Gli3C' Δ Clal were provided by Dr. Altaba (30). pBabe-BRAF^{E600} was obtained from Dr. Daniel Peeper (31).

Cell Culture and Transfection—HPDE-c7, an immortalized pancreatic ductal epithelial cell line, was provided by Dr. Ming-Sound Tsao (University of Toronto, Canada) and cultured in keratinocyte serum-free (KSF) medium supplemented with bovine pituitary extract and epidermal growth factor (Invitrogen). KRAS^{V12}-transformed HPDE-c7 cells were produced by retroviral infection of HPDE-c7 cells with a KRAS^{V12} construct. Panc-1 and AsPC-1 cells were purchased from the American Type Culture Collection (Manassas, VA) and maintained in RPMI 1640 medium (AsPC-1) or Dulbecco's modified Essential medium (Panc-1) supplemented with 10% fetal bovine serum (Invitrogen). Transfection was performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions (the ratio of plasmid (μ g) to lipid (μ l) was 1:3).

Retroviral Infection—Retroviruses were generated by transfecting amphotropic Phoenix packaging cells with the retroviral vector pBabepuro-KRAS4B^{G12V} and the corresponding empty vector pBabepuro using Lipofectamine Plus reagent (Invitrogen). Retroviral supernatants were collected, filtered, and incubated with the target cells in the presence of 4 μ g/ml Polybrene (Sigma). After 48 h, cells were subjected to selection using 0.5 μ g/ml puromycin (ICN Biomedicals, Irvine, CA) until all the untransduced cells had died.

RAS Activity Assay—The GTP loading status of RAS was assessed using a glutathione *S*-transferase (GST) fusion of the RAS binding domain (RBD) of RAF (GST-RAF-RBD) as described earlier (32).

Anchorage-independent Cell Growth Assay—Cells (1×10^3 – 5×10^5) were suspended in 2 ml of HPDE cell medium with 0.35% agarose (Invitrogen), and the suspension was placed on top of 5 ml of solidified 0.7% agarose. Triplicate cultures at three different dilutions for each cell type were maintained at 37 $^{\circ}$ C in a 5% CO₂ atmosphere, and fresh medium was added after 1 week. Colonies were photographed between 14 and 24 days under a phase contrast microscope. The number of colonies was counted from each experiment, and the experiment was reproduced twice. Student's *t* test for two samples was used to determine the statistical significance between the two groups. A *p* value less than 0.05 was regarded as statistically significant.

Immunoblotting Analysis—Protein concentration of cell lysates was assayed with the Bio-Rad protein assay reagent. Equal amounts of protein (5–30 μ g) were loaded onto 10% SDS-polyacrylamide mini-gels (Bio-Rad) and transferred to polyvinylidene difluoride membranes after electrophoresis. After being blocked overnight in 5% milk in Tris-buffered saline-Tween, blots were incubated with primary antibodies for

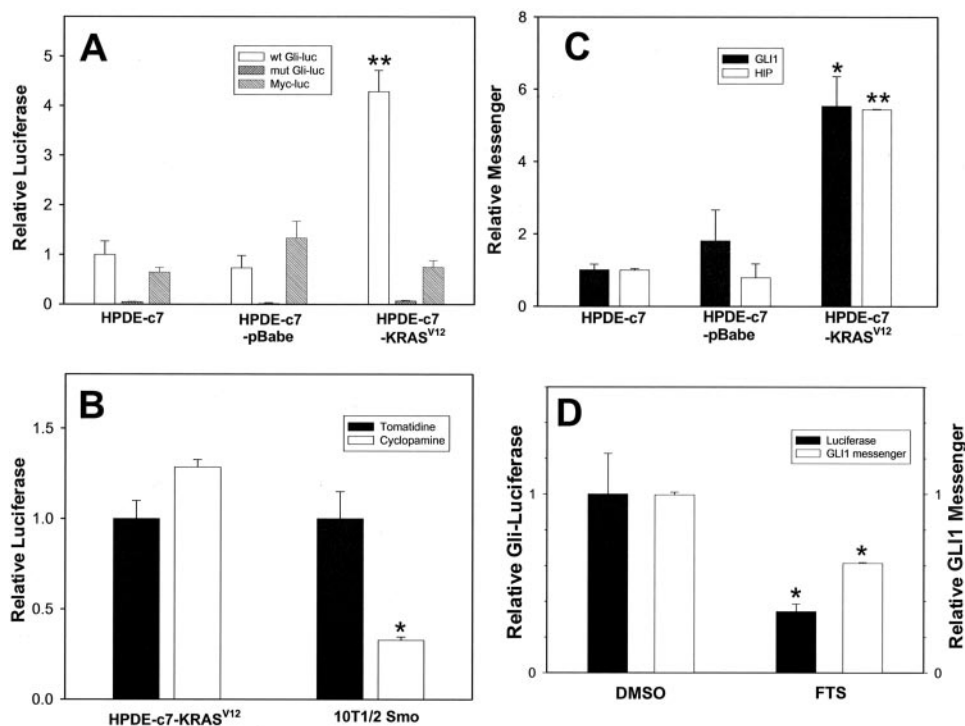


FIGURE 1. **Oncogenic KRAS activates the hedgehog signaling pathway in HPDE-c7 cells.** A, luciferase activities in HPDE-c7, HPDE-c7-pBabe, and HPDE-c7-KRAS^{V12} cells transfected with a wild-type (wt) Gli reporter, a mutant Gli reporter that does not bind Gli, or a Myc reporter. B, Gli-luciferase activities in HPDE-c7-KRAS^{V12} and 10T1/2 SMO cells treated with 5 μ M tomatidine or cyclopamine. C, expression levels of endogenous GLI1 and hedgehog-interacting protein mRNA assessed by real time PCR in HPDE-c7, HPDE-c7-pBabe, and HPDE-c7-KRAS^{V12} cells. D, Gli-luciferase activities and expression levels of endogenous GLI1 mRNA in HPDE-c7-KRAS^{V12} cells treated with FTS (2.5 μ M) for 24 h. Data are represented as the mean \pm S.E. *p* values are indicated by asterisks (relative to HPDE-c7 in A and C, to tomatidine control in B, and to Me₂SO (DMSO) control in D): single asterisk, *p* < 0.05; two asterisks, *p* < 0.01.

1.5 h followed by horseradish peroxidase-conjugated secondary antibody (1:4000) for 45 min. Antigen-antibody complexes were detected by enhanced chemiluminescence.

Luciferase Reporter Gene Assay—For luciferase assay, cells were transfected with the *Gli* or other luciferase reporter construct (0.5 μ g/well) and the SV40-*Renilla* control plasmid (2 ng/well) using Lipofectamine 2000. After 6 h the medium was replaced with fresh growth medium, and the cells were incubated in 5% CO₂ at 37 °C overnight. After treatment with U0126 or other compounds, the cells were harvested, and luciferase activity was measured with the dual luciferase reporter assay system (Promega) according to the manufacturer's instruction. Briefly, the transfected cells were lysed in the 12-well plates with 100 μ l of reporter lysis buffer, and the lysate was transferred into Eppendorf tubes. Cell debris was removed by centrifugation at top speed for 30 s in a refrigerated microcentrifuge. 20 μ l of the supernatant was mixed with 100 μ l of LAR II buffer, and the luminescence was immediately measured (first reading). After 20 s, 100 μ l of Stop & Glo[®] reagent was added to measure the *Renilla* luciferase activity (second reading). The value from the first reading was divided by the value from the second reading of each sample to obtain normalized luciferase activity. Each experiment was repeated at least three times with similar results. *Gli* luciferase reporter constructs 8 \times 3' *Gli* BSwT-luc and 8 \times 3' *Gli* BSmT-luc were provided by Dr. H. Sasaki (29).

Quantitative Real-time PCR—Total RNA was isolated from cultured cells as previously described (33). Real-time PCR analysis was conducted using an Applied Biosystems Prism 7000 sequence detection system and TaqMan[®] gene expression assays for relative quantification of *GLI1* (Hs00171790_m1) and hedgehog-interacting protein (Hs00368450_m1) mRNA. Duplicate C_T values were analyzed in Microsoft Excel using the comparative C_T($\Delta\Delta C_T$) method as described by the manufacturer (Applied Biosystems). The amount of target ($2^{-\Delta\Delta C_T}$) was obtained by normalized to endogenous reference (18 S) and relative to a calibrator.

RESULTS

Oncogenic Transformation of HPDE by KRAS^{V12}—To probe the mechanism of oncogene KRAS-mediated pancreatic tumorigenesis, we established a KRAS oncogene-based human PDA model using an immortalized primary HPDE cell line, HPDE-c7. This well characterized cell line is a near-diploid human pancreatic duct epithelial

cell line originally derived from normal pancreas. Although immortalized by E6/E7 genes of human papilloma virus-16, HPDE-c7 is non-tumorigenic and incapable of inducing tumor growth in nude mice (34, 35). Stable expression of KRAS^{V12} in HPDE-c7 cells using a retroviral expression vector led to transformation of the cell line. For all experiments performed in this study, we used early passages of the same puromycin-selected pool of KRAS^{V12}-transformed HPDE-c7 cells to eliminate potential clonal and passage variations. The resultant cells, HPDE-c7-KRAS^{V12}, expressed increased levels of total RAS protein, showed high RAS-GTP activity, and grew anchorage-independently in soft agar. In addition, expression of KRAS^{V12} in HPDE-c7 cells also led to the activation of its downstream effectors, such as MAPK and AKT. The basal phospho-MAPK and phospho-AKT levels were enhanced in the HPDE-c7-KRAS^{V12} cells compared with the parental cells. These observations are in complete agreement with results obtained from an independently established KRAS human PDA model using the same HPDE-c7 parental cell line (36).

Activation of Hh Signaling by KRAS^{V12} in HPDE—To examine the roles of the activating KRAS oncogene in controlling Hh signaling during the oncogenic transformation of HPDE, we compared the activities of Hh signaling in parental, vector control, and KRAS^{V12}-expressing HPDE cells. As shown in Fig. 1A, KRAS^{V12} expression led to a significantly increased activation of *Gli*-mediated luciferase activity,

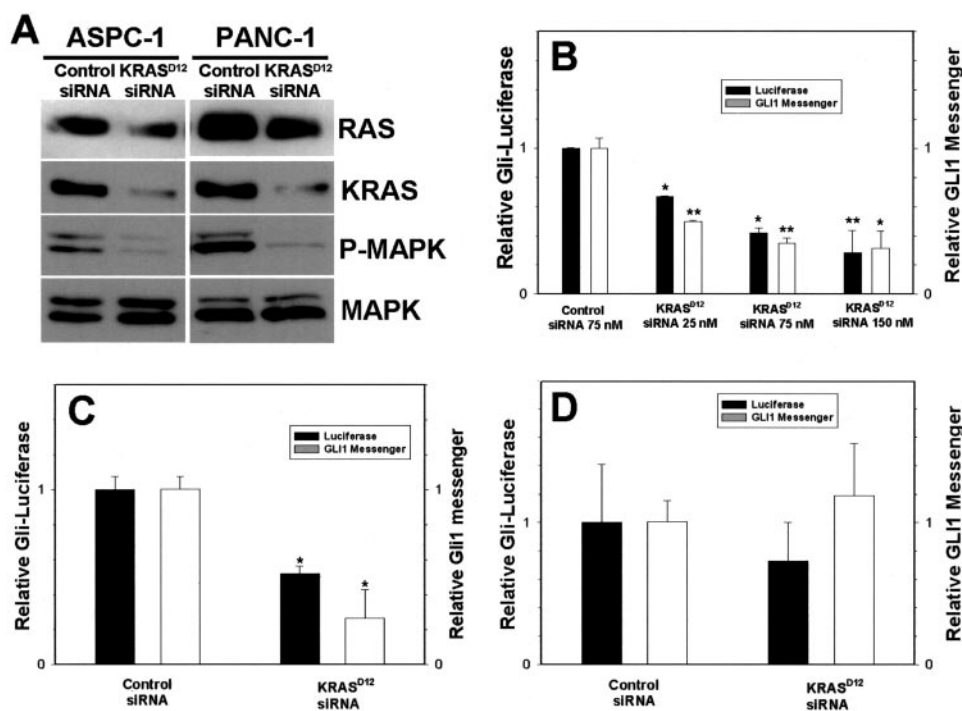


FIGURE 2. Oncogenic KRAS mediates *GLI1* activation in pancreatic cancer cells. *A*, total cellular RAS, KRAS, and phosphorylated MAPK (*P-MAPK*) levels in AsPC-1 and Panc-1 cells transfected with control and KRAS^{D12}-specific siRNAs. *B*, Gli-luciferase activities and expression levels of endogenous *GLI1* mRNA in AsPC-1 cells with KRAS^{D12} knockdown by siRNA. *C*, Gli-luciferase activities and expression levels of endogenous *GLI1* mRNA in Panc-1 cells with KRAS^{D12} knockdown by siRNA (75 μ M). *D*, Gli-luciferase activities and expression levels of endogenous *GLI1* mRNA in BxPC-3 cells treated with KRAS^{D12} siRNA (75 μ M). Endogenous *GLI1* mRNA levels are measured by real time PCR. Data are represented as the mean \pm S.E. *p* values are indicated by asterisks (relative to control): single asterisk, *p* < 0.05; two asterisks, *p* < 0.01.

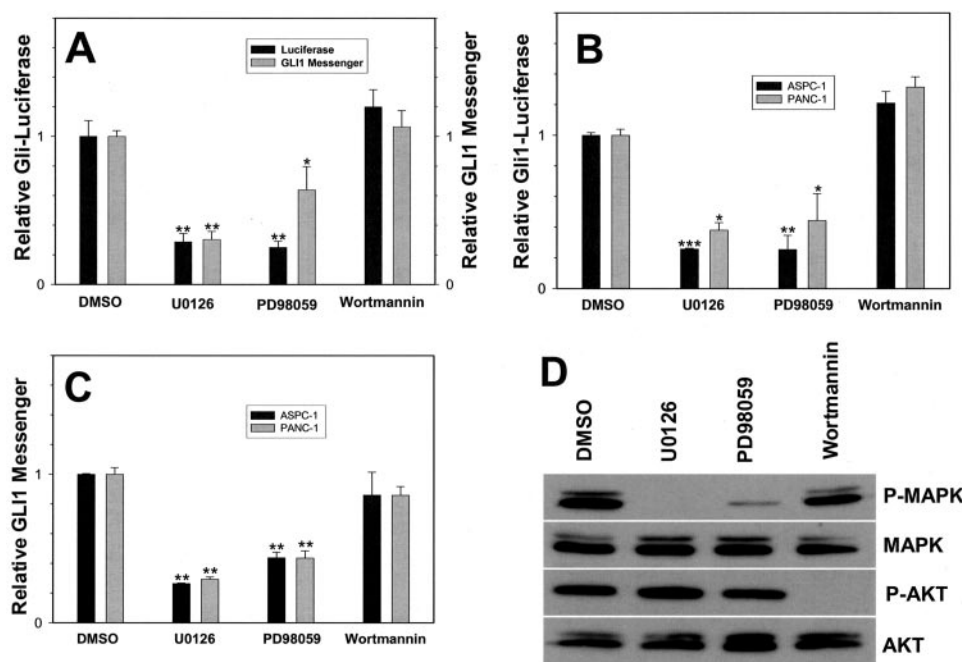


FIGURE 3. Oncogenic KRAS-induced *GLI1* activation requires MEK. *A*, Gli-luciferase activities and expression levels of endogenous *GLI1* mRNA in HPDE-c7-KRAS^{V12} cells treated with MEK-specific inhibitors U0126 and PD98059 and the PI3K-specific inhibitor wortmannin. *B*, Gli-luciferase activities in AsPC-1 and Panc-1 cells treated with MEK-specific inhibitors U0126 and PD98059 and the PI3K-specific inhibitor wortmannin. *C*, expression levels of endogenous *GLI1* mRNA in AsPC-1 and Panc-1 cells treated with MEK-specific inhibitors U0126 and PD98059 and the PI3K-specific inhibitor wortmannin. *D*, levels of phosphorylated MAPK (*P-MAPK*) and AKT (*P-AKT*) in Panc-1 cells treated with MEK-specific inhibitors U0126 and PD98059 and the PI3K-specific inhibitor wortmannin as monitored by immunoblotting analyses using anti-phospho-MAPK and anti-phospho-AKT antibodies. Endogenous *GLI1* mRNA levels are measured by real time PCR. Data are represented as the mean \pm S.E. *p* values are indicated by asterisks (relative to DMSO (DMSO) control): single asterisk, *p* < 0.05; two asterisks, *p* < 0.01; three asterisks, *p* < 0.001.

whereas the expression of the *Myc* reporter and a mutant *Gli* reporter that is incapable of binding GLI transcription factors were not affected. Moreover, the KRAS^{V12}-induced *Gli*-luciferase activity was insensitive to cyclopamine treatment (5 μ M), whereas the same treatment resulted in significant inhibition of *Gli*-luciferase activity in an SMO-expressing 10T1/2 cell line (Fig. 1*B*). These results suggest that KRAS^{V12} activates Hh signaling in HPDE cells in a ligand-independent manner. Further supporting our results based on the luciferase assay, oncogenic KRAS also up-regulated endogenous levels of *GLI1* and hedgehog-interacting protein mRNAs, both of which are Hh target genes, in immortalized HPDE cells as measured by real-time PCR (Fig. 1*C*). To ensure that the apparent activation of Hh signaling observed in HPDE-c7-KRAS^{V12} cells was caused specifically by activation of the KRAS pathway rather than an indirect effect associated with the transformation of HPDE, we suppressed the oncogenic KRAS activity in HPDE-c7-KRAS^{V12} cells using a specific nontoxic RAS antagonist, FTS, which dislodges RAS from its membrane anchorage domains and accelerates its degradation (37, 38). FTS has been shown to act as a functional KRAS inhibitor in human pancreatic cell lines that express activated KRAS (39). As shown in Fig. 1*D*, treatment of HPDE-c7-KRAS^{V12} with FTS led to an inhibition of *Gli*-luciferase and endogenous *GLI1* mRNA. Taken together, our studies suggest that KRAS^{V12} specifically activates Hh signaling in HPDE cells as *GLI1* is not only a downstream effector but also a direct target gene and a reliable marker of Hh signaling pathway activities (40).

Oncogenic KRAS Is Responsible for *GLI1* Activation in PDA Cells—To further test whether oncogenic KRAS is essential for *GLI1* activation in PDA cells, we knocked down the expression of the onco-

KRAS Activates Hedgehog Signaling

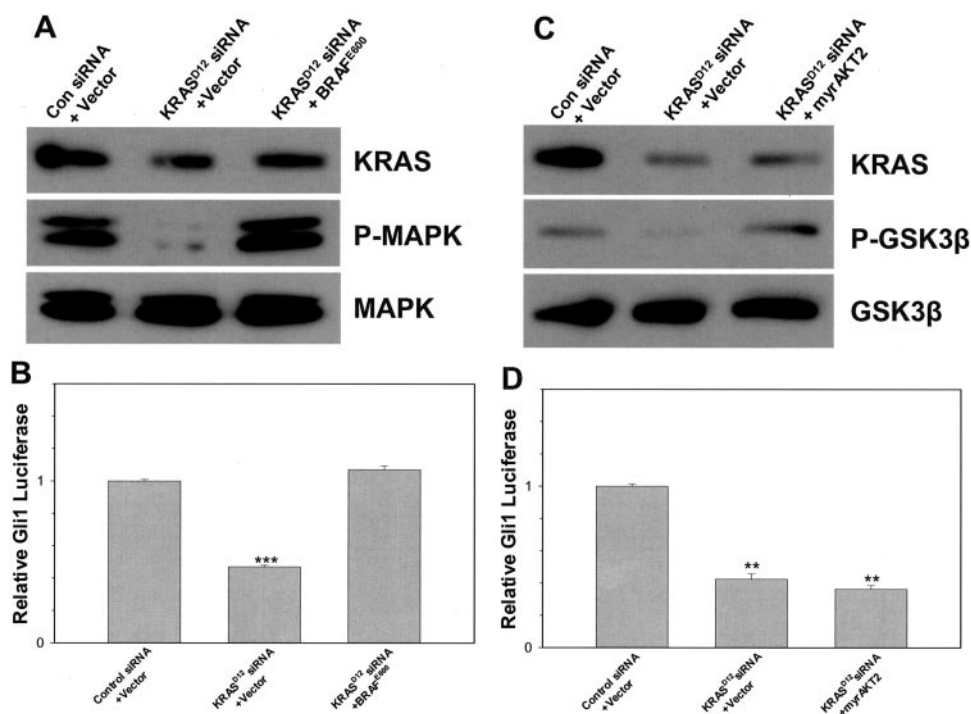


FIGURE 4. Constitutively active BRAF^{E600} rescues Gli activation in KRAS^{D12} knockdown AsPC-1 cells. *A*, total cellular KRAS, phosphorylated MAPK (*P-MAPK*) and total MAPK levels in AsPC-1 cells co-transfected with control or KRAS^{D12}-specific siRNA and control or BRAF^{E600} expression vector. *B*, Gli-luciferase activities in AsPC-1 cells co-transfected with control or KRAS^{D12}-specific siRNA and control or BRAF^{E600} expression vector. *C*, total cellular KRAS, phosphorylated glycogen synthase kinase 3β (*P-GSK3β*), and total GSK3β levels in AsPC-1 cells co-transfected with control or KRAS^{D12}-specific siRNA and control or myr-AKT2 expression vector. *D*, Gli-luciferase activities in AsPC-1 cells co-transfected with control or KRAS^{D12}-specific siRNA and control or myr-AKT2 expression vector. Data are represented as mean ± S.E. *p* values are indicated by asterisks (relative to control): **, *p* < 0.01; ***, *p* < 0.001.

genic KRAS in human PDA cell lines (AsPC-1 and Panc-1) that express mutant KRAS^{D12} using RNA interference. A previously reported siRNA duplex that specifically knocks down KRAS^{D12} in AsPC-1 and Panc-1 cells was used (41). The inactivation of KRAS oncogene by the KRAS^{D12}-specific siRNA duplex in AsPC-1 and Panc-1 cells led to decreased cellular RAS levels (Fig. 2*A*). Consistent with the reduced KRAS level, the phospho-MAPK levels were suppressed in KRAS^{D12}-knockdown AsPC-1 and Panc-1 cells compared with cells treated with the control siRNA, confirming the specificity and effectiveness of KRAS^{D12}-siRNA. Gene silencing of KRAS^{D12} in AsPC-1 and Panc-1 cells eventually led to growth inhibition and cell death 96 h after siRNA transfection. Therefore, experiments related to KRAS^{D12} siRNA were performed 24 h after transfection when no noticeable difference between control and KRAS^{D12} siRNA-transfected cells could be observed. KRAS^{D12} knockdown by siRNA resulted in a dose-dependent inhibition of *Gli*-luciferase activity and endogenous levels of *GLI1* mRNA in AsPC-1 (Fig. 2*B*). Similar results were also observed in Panc-1 cells (Fig. 2*C*). To exclude the possibility that our observed inactivation of *Gli*-luciferase by KRAS^{D12}-specific siRNA was due to potential off-target effects, we used a PDA cell line, BxPC-3 cells (which does not contain KRAS mutation), to confirm the specificity of KRAS^{D12} siRNA. As expected, the *Gli*-luciferase activity and *GLI1* mRNA level in BxPC-3 cells did not change significantly after introducing KRAS^{D12}-specific siRNA (Fig. 2*D*). These results demonstrate

that effects of KRAS^{D12} siRNA observed in AsPC-1 and Panc-1 cells are specific to the KRAS^{D12} mutant allele. Taken together, our data indicate that oncogenic KRAS plays an important role in regulating Hh signaling in PDA cells.

RAF/MEK/MAPK, but Not the PI3K/AKT Pathway, Is Required for the KRAS-mediated Activation of Hh Signaling—To further determine which downstream effectors of the oncogene KRAS mediate the activation of Hh signaling, we examined the levels of Hh activity in HPDE-c7-KRAS^{V12} cells in response to specific inhibitors that target the RAS downstream effectors MEK and PI3K. Inhibition of MEK by U0126 (20 μM) and PD98059 (40 μM) led to a significant reduction of *Gli*-luciferase activity and endogenous *GLI1* mRNA levels (Fig. 3*A*), whereas wortmannin (100 nM), a PI3K-specific inhibitor, had little effect. These results suggested that the RAF/MEK/MAPK pathway was directly responsible for the KRAS-mediated activation of Hh signaling in HPDE-c7-KRAS^{V12} cells. The inhibitory effect of U0126 and PD98059 was also observed in PDA

cells expressing activating KRAS mutant. When AsPC-1 and Panc-1 cells were treated with the various inhibitors for 24 h, U0126 and PD98059 inhibited *Gli*-luciferase expression (Fig. 3*B*) as well as endogenous *GLI1* mRNA levels (Fig. 3*C*), whereas wortmannin had no effects. To ensure that the apparent non-effect of wortmannin is not due to a lack of efficacy of the compound, we determined the phosphorylation status of MAPK and AKT in Panc-1 cells treated with various pharmacological inhibitors. As shown in Fig. 3*D*, U0126 and PD98059 significantly suppressed the phosphorylation levels of MAPK. Wortmannin abolished the phosphorylation of Ser-473 of AKT, which is essential for its activation.

If the RAF/MEK/MAPK, but not the PI3K/AKT pathway, is indeed responsible for the KRAS-mediated activation of Hh signaling, we expect that expressing a constitutively active RAF kinase, but not a constitutively active AKT, will rescue the inhibitory effect of oncogene KRAS knockdown on Hh activation. To confirm our study using pharmacological inhibitors, we co-transfected either BRAF^{E600}, a constitutively active BRAF mutant, or myrAKT2, a membrane-targeted, constitutively active form of AKT2, together with the KRAS^{D12}-specific siRNA duplex into AsPC-1 and subsequently monitored the *Gli*-luciferase activity. As shown in Fig. 4*A*, expression of BRAF^{E600} restored KRAS^{D12}-specific siRNA-mediated suppression of MEK activity and completely blocked the inhibitory effect of KRAS^{D12}-specific siRNA on Hh signaling (Fig. 4*B*). On the other hand, although myr-AKT2 brought back the AKT

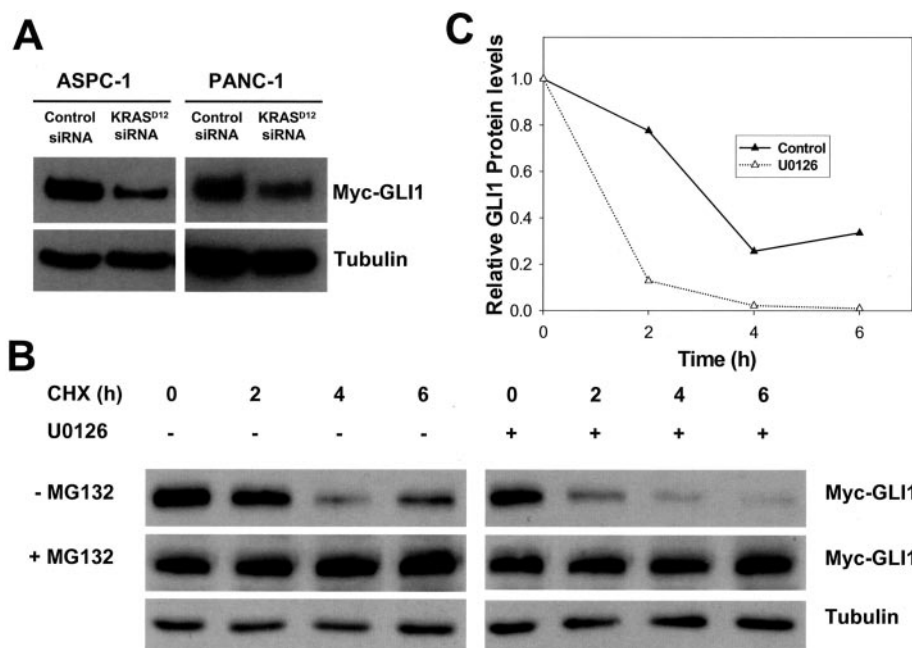


FIGURE 5. Suppression of KRAS/RAF/MEK/MARK pathway decreases GLI1 protein stability in PDA cells. *A*, levels of ectopically expressed GLI1-Myc proteins in AsPC-1 and Panc-1 cells transfected with control and KRAS^{D12}-specific siRNAs. *B*, levels of ectopically expressed GLI1-Myc proteins in Panc-1 treated with protein synthesis inhibitor cycloheximide (CHX, 50 μ g/ml) in the presence or absence of U0126 (20 μ M) and MG132 (30 μ M) as a function of time. *C*, densitometer quantification of data in *B* in the absence of MG132 to determine the effect of U0126 on GLI1-Myc proteins half-life.

activity inhibited by KRAS^{D12}-siRNA, as measured by glycogen synthase kinase 3 β (GSK3 β) phosphorylation (Fig. 4C), expression of myr-AKT2 failed to rescue *Gli*-luciferase activity brought down by KRAS^{D12}-siRNA (Fig. 4D). Similar results were obtained using Panc-1 cells (data not shown). These results indicate that the RAF/MEK/MAPK signaling, but not the PI3K/AKT pathway, is critical for KRAS-mediated *GLI* activation in PDA.

KRAS, through the RAF/MEK/MAPK Pathway, Regulates GLI1 Protein Degradation—To further elucidate the mechanism by which oncogene KRAS mediates activation of Hh signaling, we determine whether oncogenic KRAS could regulate GLI1 at the protein level. Because none of the commercially available GLI-antibodies was specific or sensitive enough to monitor the endogenous GLI protein levels, we co-transfected an Myc-tagged GLI1 expression vector (42) and KRAS^{D12}-specific siRNA duplex into AsPC-1 and Panc-1 cells and subsequently monitored the expression levels of GLI1 24 h post-transfection using anti-Myc antibody. As shown in Fig. 5A, the expression levels of GLI1 were significantly reduced in both AsPC-1 and Panc-1 cells with oncogenic KRAS activity silenced by siRNA. After inhibition of new protein synthesis by cycloheximide (50 μ g/ml), we observed that inhibition of MEK by U0126 led to a significant reduction of GLI1 stability in Panc-1 cells (Fig. 5B). The apparent half-life of GLI1 protein in untreated Panc-1 cells was about 3 h, whereas the half-life of GLI1 protein in U0126-treated Panc-1 cells was estimated to be less than 1 h (Fig. 5C). Furthermore, GLI1 protein degradation was blocked by the proteasome inhibitor MG132 in the absence or presence of U0126. Taken together, these results suggest the oncogene KRAS, through the RAF/MEK/MAKP pathway,

blocks proteasome-mediated GLI1 degradation and consequently leads to the activation of Hh signaling in pancreatic cancer cells.

Suppression of GLI Activity Attenuates the Transforming Activity of KRAS Oncogene—To investigate the physiological relevance and contribution of KRAS-mediated Hh signaling activation in pancreatic cancer development, we suppressed GLI1 expression using an established *GLI1*-specific siRNA (43). The specificity of this particular *GLI1* siRNA has been extensively demonstrated previously (43). To test the effectiveness of the *GLI1* siRNA, we co-transfected a Myc-tagged GLI1 construct with control or *GLI1* siRNA into AsPC-1 and Panc-1 cells. *GLI1* siRNA effectively suppressed GLI1 protein expression in Panc-1 cells (Fig. 6A, inset). Although *GLI1* siRNA does not affect growth or apoptosis of AsPC-1 and Panc-1 cells under typical tissue culture growth conditions (supplemental Fig. S1), *GLI1* gene silencing led to a significant attenuation of anchorage-independent growth of Panc-1 cells as measured by the soft agar colony formation assay (Fig. 6A). Similar results were observed using AsPC-1 cells (data not shown). To further confirm this observation, we ectopically expressed a C-terminal-truncated Gli3 construct in AsPC-1 and Panc-1 cells. This specific deletion mutant, Gli3C' Δ ClaI, has been demonstrated to exert strong dominant negative effects on Hh signaling both *in vitro* and *in vivo* (30). As expected, expressing of Gli3C' Δ ClaI led to attenuated Hh signaling as indicated by the apparent reduction of *Gli*-luciferase activity in AsPC-1 cells (Fig. 6B). Moreover, expression of Gli3C' Δ ClaI significantly suppressed anchorage-independent growth of AsPC-1 in a manner similar to that of *GLI1* siRNA (Fig. 6C). We observed similar results in Panc-1 cells (data not shown). In contrast, *GLI1* gene silencing had no significant effect on anchorage-independent growth of BxPC-3 cells, which contain no KRAS mutation (Fig. 6D). Taken together, these data suggest that activation of Hh signaling is important for KRAS oncogenic transformation in pancreatic cancer cells.

blocks proteasome-mediated GLI1 degradation and consequently leads to the activation of Hh signaling in pancreatic cancer cells.

DISCUSSION

Constitutively active KRAS mutations are one of the earliest and most common genetic alterations in pancreatic carcinomas (13). Cross-talk between KRAS and other oncogenic pathways in PDA have not been investigated extensively. In this study we explored the cross-talk between oncogenic KRAS and the Hh signaling pathway, which has recently been shown to be up-regulated in human PDA tissues and cell lines (21–23). In the immortalized human pancreatic ductal epithelial cell line, HPDE-c7, we demonstrated that expressing KRAS^{V12} led to

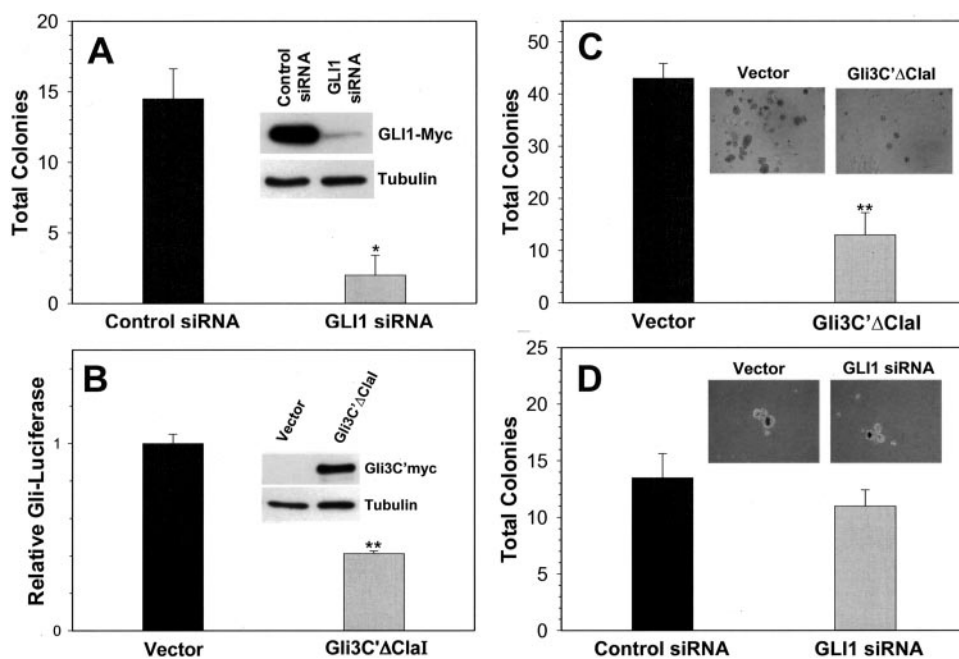


FIGURE 6. Down-regulation of GLI activity suppresses anchorage-independent growth of PDA cells with oncogene KRAS. *A*, anchorage-independent growth potential of Panc-1 cells transfected with control or *GLI1*-specific siRNAs tested by soft agar assay. *Inset*, levels of ectopically expressed *GLI1*-Myc proteins in Panc-1 transfected with control and *GLI1*-specific siRNAs. *B*, Gli-luciferase activities in AsPC-1 cells transfected with empty vector or *Gli3C'ΔClal* construct. *Inset*, levels of ectopically expressed dominant negative *Gli3* deletion mutant, *Gli3C'ΔClal*, in AsPC-1 transfected with empty vector and *Gli3C'ΔClal* construct. *C*, anchorage-independent growth potential of AsPC-1 cells transfected with control or *Gli3C'ΔClal* vector tested by soft agar assay. *D*, anchorage-independent growth potential of BxPC-3 cells transfected with control or *GLI1*-specific siRNAs tested by soft agar assay. Data, represented as the mean \pm S.E. *p* values, are indicated by asterisks (relative to control): one asterisk, $p < 0.05$; two asterisks, $p < 0.01$.

increased endogenous *GLI1* levels and transcriptional activity, whereas suppressing oncogenic *KRAS* expression by siRNA inhibits *GLI* activity and *GLI1* expression in PDA cell lines with activating *KRAS* mutation. Furthermore, we found that *KRAS*-mediated Hh activation was suppressed by pharmacological inhibitors specific for MEK, but not PI3K, in *KRAS*-transformed HPDE and mutant *KRAS* expressing PDA cell lines. Expression of constitutive active form of *BRAF*^{E600}, but not myr-AKT, blocks the inhibitory effects of *KRAS* knockdown on Hh signaling. Our studies suggest that oncogenic *KRAS*, through the RAF/MEK/MAPK pathway, suppresses *GLI1* protein degradation and consequently plays an important role in activating the Hh signaling pathway in the absence of additional Hh ligand during pancreatic tumorigenesis.

Although biochemical and genetic analyses suggest that three *GLI* transcription factor isoforms (*GLI1*, *GLI2*, and *GLI3*) play distinct as well as overlapping roles in mammalian Hh signal transduction during development (44), the individual roles of *GLI1*, *GLI2*, and *GLI3* in pancreatic tumorigenesis are not very well understood. Overexpression of *GLI1*, along with other Hh signaling molecules such as Sonic hedgehog (Shh), Indian hedgehog, SMO, and *PATCH*, have been reported in human pancreatic cancer tissues and pancreatic cancer cell lines (21–23). On the other hand, the status of *GLI2* in human pancreatic cancer has not been extensively studied. A recent study of a transgenic mice based on a dominant active form of the *Gli2* transcription factor suggest that although *Gli2* activation is sufficient to drive pancreatic neoplasia, it does not

recapitulate human pancreatic carcinogenesis (28). Considering the extensive sequence and structure homology between *GLI1* and *GLI2*, it is conceivable that these proteins are regulated by RAS-RAF-MAPK signaling pathway in a similar manner. Indeed, our preliminary studies suggest that expression of *GLI2* is also inhibited by *KRAS* siRNA knockdown and MEK inhibitors, just like *GLI1* expression in both AsPC-1 and Panc-1 cells (supplemental Fig. S2).

Our findings provide direct evidence that oncogenic *KRAS* activates Hh signaling through up-regulation of *GLI* in PDA. Although deregulation of Ras signaling in mice lacking *p53* function in the pancreatic epithelium has recently been shown to induce Shh expression (20), we believe that ligand-independent activation of the Hh pathway represents another important mechanism by which oncogenic *KRAS* promotes tumor formation. The ability of oncogenic *KRAS* to activate Hh signaling in the absence of Hh ligand also offers an

explanation for why more than 50% of PDA cells lines with sustained Hh signaling activity are resistant to cyclopamine (21). The present findings combined with an earlier observation that sustained Hh activation activates platelet-derived growth factor receptor α and the RAS pathway (45) suggest that RAS and Hh signaling pathways can potentially form a positive feedback loop to promote tumorigenesis in pancreatic cancer.

Although PI-3K and AKT activities are essential for ligand-dependent Shh signaling in the specification of neuronal fates in chicken neural explants and chondrogenic differentiation of 10T1/2 cells, activation of the phosphoinositide 3-kinase/AKT pathway alone is not sufficient to drive *Gli* activation (46). Our study indicates that activation of the RAS pathway is sufficient to drive Hh signaling activation in PDA cells through up-regulation of *GLI* expression without the addition of Hh ligand, and the effect of RAS is mediated exclusively by the RAF/MEK/MAPK pathway independent of PI3K/AKT. Furthermore, suppression of *GLI1* expression in oncogenic *KRAS*-containing PDA cells leads to a significant attenuation of anchorage-independent growth in soft agar, suggesting that activation of the Hh signaling pathway may represent an important mechanism for *KRAS*-mediated pancreatic tumorigenesis.

Our study is consistent with a recent report by Hebrok and co-workers (28), showing that although Hh signaling alone does not recapitulate human pancreatic carcinogenesis, cooperation of Hh and Ras signaling significantly reduces the latency of PanIN formation in mice simultaneously expressing dominant active *Gli2* and *Kras*^{D12} compared with transgenic mice

expressing Kras^{D12} alone. Our results are also in agreement with an earlier observation in which phorbol ester, acting through protein kinase C δ and the extracellular signal-regulated kinase pathway, can stimulate Gli transcriptional activity in NIH3T3 cells in a ligand-independent manner (47). Taken together, these observations and our findings suggest that activation of the RAS/MAPK pathway, induced by diverse upstream signals and converging at the level of GLI transcription factors, is an integral component of the Hh signaling. The realization of cross-talk between RAS/MAPK and Hh signaling pathways in pancreatic carcinomas also suggests that targeting the RAS and Hh pathways synergistically may represent a new therapeutic strategy for PDA.

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