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Phosphorylation at Ser-181 of Oncogenic KRAS Is Required for Tumor Growth

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Abstract
KRAS phosphorylation has been reported recently to modulate the activity of mutant KRAS protein in vitro. In this study, we defined S181 as a specific phosphorylation site required to license the oncogenic function of mutant KRAS in vivo. The phosphomutant S181A failed to induce tumors in mice, whereas the phosphomimetic mutant S181D exhibited an enhanced tumor formation capacity, compared with the wild-type KRAS protein. Reduced growth of tumors composed of cells expressing the nonphosphorylatable KRAS S181A mutant was correlated with increased apoptosis. Conversely, increased growth of tumors composed of cells expressing the phosphomimetic KRAS S181D mutant was correlated with increased activation of AKT and ERK, two major downstream effectors of KRAS. Pharmacologic treatment with PKC inhibitors impaired tumor growth associated with reduced levels of phosphorylated KRAS and reduced effector activation. In a panel of human tumor cell lines expressing various KRAS isoforms, we showed that KRAS phosphorylation was essential for survival and tumorigenic activity. Furthermore, we identified phosphorylated KRAS in a panel of primary human pancreatic tumors. Taken together, our findings establish that KRAS requires S181 phosphorylation to manifest its oncogenic properties, implying that its inhibition represents a relevant target to attack KRAS-driven tumors. Cancer Res; 74(4); 1190–9. © 2013 AACR.

Introduction
RAS proteins are well-known small GTPases involved in the regulation of key signal transduction pathways. Cycling from the inactive (GDP-bound) to the active (GTP-bound) state faithfully responds to extracellular signals due to its tight regulation by GTP exchange factors (GEF) and GTPase activating proteins (GAP). Activating point mutations that render RAS proteins insensitive to the extracellular signals are crucial steps in the development of the vast majority of cancers (1–3). Three different genes code for a total of four different Ras isoforms named HRAS, NRAS, KRAS4A and KRAS4B. RAS mutations, mainly at the KRAS4B (herein after referred to as KRAS) genes, occur in pancreatic (95%), colon (40%) and adenocarcinomas of the lung (35%; refs. 1, 4, 5). The most prevalent oncogenic mutations in RAS at codons 12, 13 and 61 preserve the GTP-bound, active state by inhibiting intrinsic GTPase activity or interfering with the action of GAPs. In the GTP-bound form, RAS is able to interact with different effector proteins and consequently activates signal transduction pathways. Among those, the best characterized are the RAF1/MEK/ERK and the phosphatidylinositol-3-kinase (PI3K)/AKT (6, 7).

Since oncogenic mutations of KRAS give rise to an always GTP-bound protein that constitutively activates the effectors, positive or negative physiological regulation of oncogenic KRAS was not initially expected. Several reversible postranslational modifications of KRAS have been described that could modulate KRAS oncogenic activity (8). Ubiquitination of oncogenic KRAS at lysine-147 in the guanine nucleotide-binding motif increases its binding to the downstream effectors PI3K and RAF1 thus increasing its tumorigenic activity (9). Furthermore, acetylation at lysine-104 affects interaction with GEFs and inhibits in vitro transforming activity of oncogenic KRAS (10). KRAS has, adjacent of the farnesylated C-terminal cysteine, a stretch of six contiguous lysines in a total of eight lysine residues, known as the polybasic domain, which promotes an electrostatic interaction with the negatively charged phosphate groups of phospholipids (11, 12). Phosphorylation of KRAS at serine-181 within this domain has been described (13). We previously

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reported a role of KRAS Ser181 phosphorylation for activation of the wild-type KRAS in vitro and to regulate also in vitro oncogenic KRAS activity (14). By using both a genetic and pharmacologic approach, we demonstrate here that phosphorylation of oncogenic KRAS is required for tumor growth in vivo and that also this modification can be detected in human tumors. Furthermore, pharmacologic inhibition of oncogenic KRAS phosphorylation suppresses KRAS oncogenic activity.

Materials and Methods

Antibodies and reagents

Primary antibodies used for immunoblotting were as follow: Anti-Actin (clone C4; #619010, 1:1000; MP Biomedicals), Anti-GAPDH (#MAB374, 1:1000; Chemicon); Anti-cleaved caspase-3 (Asp175; #9661, 1:1000; Cell Signaling Technology); Anti-akt (#9272; Cell Signaling Technology); Anti-phospho-akt (Thr308; #9275, 1:1000; Cell Signaling Technology), Anti-p44/42 MAPK (ERK 1/2; #9102, 1:1000; Cell Signaling Technology); Anti-phospho-p44/42 MAPK (ERK 1/2; Thr202/ Tyr204; #9101, 1:1000; Cell Signaling Technology); Anti-cyclin B1 (#4138, 1:1000; Cell Signaling Technology); Anti-KRAS (clone Ab-1) mouse (#OP24, 1:400; Calbiochem); Anti-Pan-Ras (clone Ab-3) mouse (#OP40, 1:400; Calbiochem); Anti-HRAS (clone Ab-1) mouse (#OP24, 1:400; Calbiochem); Anti-NRAS (clone F155) mouse (#Sc-31, Santa Cruz Biotechnology); Anti-GAP120 (sc-63, 1:100; Santa Cruz Biotechnology); Anti-phospho-p44/42 MAPK (ERK 1/2; Thr202/ Tyr204; #9101, 1:1000; Cell Signaling Technology), Anti-cyclin B1 (#4138, 1:1000; Cell Signaling Technology); Anti-KRAS (clone Ab-1) mouse (#OP24, 1:400; Calbiochem); Anti-Pan-Ras (clone Ab-3) mouse (#OP40, 1:400; Calbiochem); Anti-HRAS (clone Ab-1) mouse (#OP24, 1:400; Calbiochem); Anti-NRAS (clone F155) mouse (#Sc-31, Santa Cruz Biotechnology); Anti-GAP120 (sc-63, 1:100; Santa Cruz Biotechnology); Anti-PKCo (#10398, 1:500; BD Transduction Laboratories); Anti-phospho-PKCo (Ser643/676; #9376, 1:1000; Cell Signaling Technology). For immunohistochemistry, we used Anti-Ki-67 (SP6; #NM-9106S, 1:200; NeoMarkers). We used DeadEnd Fluorometric TUNEL System (G7132, Promega) for the terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) assays.

The reagents used for the detection of phosphorylated KRAS were: Protein phosphatase λ (#539514-20K; Calbiochem); Phos-tag (#AA1-107, Wako Chemicals GmbH).

The inhibitors of PKC used were: Bryostatin-1 (#BIB0342, Apollo Scientific), Edelfosine (1-O-Octadecyl-2-O-methylglycerol-3-phosphorylcholine; #BML-L108, Enzo Life Science), Bisindolylmaleimide I (BIM; #610398, 1:500; BD Transduction Laboratories); Anti-phospho-PKCo (Ser643/676; #9376, 1:1000; Cell Signaling Technology). For immunohistochemistry, we used Anti-Ki-67 (SP6; #NM-9106S, 1:200; NeoMarkers). We used DeadEnd Fluorometric TUNEL System (G7132, Promega) for the terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) assays.

Cell lines

NIH3T3, SW-480, A549, MPane-96, and HPAF-II cells obtained from American Tissue and Cell Collection (ATCC) were grown in Dulbecco’s Modified Eagle Medium (DMEM) containing 10% fetal calf serum (FCS; Biological Industries), and routinely verified according to the specifications outlined in the ATCC Technical Bulletin. NIH3T3 stable cell lines expressing either HA-KRAS-G12V, HA-KRAS-G12V-S181A, or HA-KRAS-G12V-S181D were obtained as previously described (14).

DLD-1 knockout of mutant KRAS allele DLD1KRASwt/− were obtained from Horizon Discovery Ltd. (clone D-WT7, #HD105-002; http://www.horizondiscovery.com). DLD1KRASwt/− cells were generated using the proprietary adeno-associated virus (AAV) gene targeting technology GENESIS. Cells were maintained according to the supplier’s recommendations in McCoy’s modified media containing 10% FBS (Biological Industries). DLD1KRASwt/− stable cell lines expressing either HA-KRAS-G12V, HA-KRAS-G12V-S181A, or HA-KRAS-G12V-S181D were obtained from DLD1KRASwt/− after transfecting with the specific HA-KRAS-G12V plasmids (14) and a puromycine resistance plasmid (pSG5A). After selection with puromycin (4 μg/mL) clones or pools were obtained.

Tumor generation in mice

The day of the injection, one million NIH3T3 cells stably expressing either HA-KRAS-G12V, HA-KRAS-G12V-S181A, or HA-KRAS-G12V-S181D suspended in 0.1 mL PBS buffer were subcutaneously injected into both flanks of Swiss nude mice (foxn1−/−). Generated tumors were measured over time and at day 18 after injection, mice were euthanized and tumors were harvested, weighed, measured, and processed for analysis (each group n = 10).

For DLD-1 xenografts, one million cells stably expressing either HA-KRAS-G12V or HA-KRAS-G12V-S181D suspended in 0.1 mL PBS buffer were subcutaneously injected into both flanks of Swiss nude mice (foxn1−/−). Generated tumors were measured over time and at day 28 after injection, mice were euthanized and tumors were harvested, weighed, measured, and processed for analysis (each group n = 10).

For the assays with the PKC inhibitors, one million NIH 3T3 cells stably expressing either HA-KRAS-G12V or HA-KRAS-G12V-S181D were subcutaneously injected into both flanks of Swiss nude mice. When tumor reached a designated volume of approximately 150 mm3, animals were randomized and divided into vehicle (dimethyl sulfoxide; DMSO), Bryostatin-1, or Edelfosine treatment groups. Mice were weighed daily and received an intraperitoneal injection of either 75 μg/kg Bryostatin-1 in 5% (v/v) DMSO, 30 mg/kg Edelfosine in 5% (v/v) DMSO, or 5% (v/v) DMSO (vehicle) for 5 days. At day 5 after the beginning of the treatment, mice were euthanized and tumors were harvested, weighed, measured, and processed for analysis.

All mouse experiments were performed in accordance with protocols approved by the Animal Care and Use Committee of ICO-IDIBELL Hospital de Llobregat (Barcelona, Spain).

Sample lysis, gel electrophoresis, and immunoblotting

Cultured cells were lysed in Ras extraction buffer (20 mmol/L Tris–HCl, pH 7.5; 2 mmol/L EDTA, 100 mmol/L NaCl, 5 mmol/L MgCl2, 1% (v/v) Triton X-100, 5 mmol/L NaF, 10% (v/v) glycerol, and 0.5% (v/v) 2-mercaptoethanol) supplemented with a cocktail of protease and phosphatase inhibitors (0.1 mmol/L Na2VO4, 1 mmol/L phenylmethylsulfonyl fluoride, 10 mmol/L β-glycerophosphate, 2 μg/mL aprotinin, and 10 μg/mL leupeptin). Tumors were lysed using Polytron (Fischer Scientific) in Ras extraction buffer and protein resolved using standard SDS–PAGE. After electrotransfer, membranes were incubated using the indicated antibodies and then incubated with peroxidase-coupled secondary antibody. Immunocomplexes were detected by enhanced chemiluminescence reaction (ECL-Western Blotting Analysis System (Amersham Biosciences) and
imaged by LAS-3000 (Fujifilm). When required, band intensity was determined using the measurement tool of Multigauge 2.0 (FUJIFILM).

Cell viability assay (MTT)
Cells lines were seeded in 96 well plates at 10^4 cell per well with DMEM 10% FCS. The next day, they were treated with the corresponding concentration of PKC inhibitors for 48 hours. Then, 10 μL of AB solution (MTT Cell Growth Assay Kit; #CT02, Millipore) were added to each well and incubated at 37°C for 4 hours. Then, 0.1 mL isopropanol with 0.04 N HCl was added and mixed thoroughly. Absorbance was measured with a test wavelength of 570 nm and a reference wavelength of 630 nm according to manufacturer’s recommendations.

Measurement of Ras isoform activation
RBD (Ras-binding domain of Raf-1) pull-down assays were performed as previously described (14) to determine the amount of active K-, H-, and NRAS.

Histology
Mice tumors were embedded either in paraffin or frozen in optimum cutting temperature (OCT). Paraffin sections were stained following the haematoxylin-eosin standard protocol to study their histologic appearance. Mitotic count in 5 consecutive high-power fields (>100) was performed to compare the mitotic index between groups. Frozen section in OCT were used to determine apoptosis by TUNEL assay following manufacturer’s recommendations (Roche) and to determine the percentage of proliferating cells by immunohistochemistry using Ki-67 antibodies.

Human tumors
Five biopsies of human pancreatic ductal adenocarcinoma obtained by duodenopancreatectomy were orthotopically implanted to nude mice and were perpetuated at least four passages. All patients gave informed written consent to participate and to have their biologic specimens analyzed. The study was cleared by the Ethical Committee of Hospital de Bellvitge.

Detection of phospho-KRAS
Phos-tag SDS–PAGE: To detect phospho-KRAS from human tumor samples and from nude mice grafts, a fragment of approximately 0.1 g from a tumor biopsy was homogenized in 0.4 mL of Phosphatase Lysis Buffer (50 mmol/L Tris-HCl pH 8; 150 mmol/L NaCl, 2 mmol/L EDTA, 10% glycerol, 1% Nonidet P40, 5 mmol/L DTT, 2 mmol/L MnCl₂) containing either protease inhibitor cocktail (Halt Protease Inhibitor Cocktail, #78786, Thermo Scientific) alone or plus phosphatase inhibitors (0.2 mmol/L Na₃VO₄, 5 mmol/L NaF). For human cell lines, a 10 cm dish was homogenized in the Phosphatase Lysis Buffer as described above. Then, samples homogenized with only protease inhibitors were treated with recombinant Protein Phosphatase λ for 30 minutes at 30°C according to manufacturer instructions, and finally all tubes were balanced with phosphatase inhibitors to equalize both lysis buffers. Protein content was assessed by the Lowry method (15) and tubes were balanced. Ten micrograms of protein were loaded into a 12%-polyacrylamide SDS–PAGE gel supplemented with...
100 μmol/L Phos-tag and 100 μmol/L MnCl$_2$ (according to Phos-tag SDS–PAGE protocol indicated by manufacturers). The gel was run overnight at 5 mA/gel and soaked in a general transfer buffer containing 1 mmol/L EDTA for 20 minutes followed by 10 minutes incubation with a transfer buffer without EDTA. Then, gels were transferred overnight at 50 V into a polyvinylidene difluoride membrane that was blocked and blotted with anti-KRAS (#OP24, Calbiochem).

Two-dimensional gel electrophoresis. One-hundred micrograms of tumor extract prepared as indicated above were diluted to a final concentration of 7 mol/L urea, 2 mol/L thiourea, 4% CHAPS {3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate}, 65 mmol/L DTE, 0.1% ampholytes (Bio-Lyte 3/10, no. 163-1113; Bio-Rad), and 1.2% Destreak Reagent (GE Healthcare, 17-6003-18) to 125 μL volume. Two-dimensional first-dimension electrophoresis was performed as isoelectric focusing (IEF) with precast, immobilized pH gradient (IPG) gel strips [ReadyStripTM IPG Strip, 7 cm, pH 7–10; no. 163-2005 (Bio-Rad)] by using a PROTEAN IEF system (Bio-Rad). Sample application and rehydration of the strips were carried out using the active method (50 V constant) according to the manufacturer’s instructions (Bio-Rad). Next focusing was performed at 8,000 to 20,000 V per hour. IEF gels were equilibrated for 10 minutes in a buffer containing 6 mol/L urea, 0.375 mol/L Tris (pH 8.8), 2% SDS, 20% glycerol, and 2% (w/v) DTE, and the second-dimension run was carried out in SDS-polyacrylamide gels. After electrophoresis, gels were transferred to polyvinylidene difluoride membranes (Millipore) and immunoblotted with antibodies against KRAS.

Statistics
All analyses were performed with GraphPad Prism 5.0. Data represent mean ± SEM. Mann–Whitney test was used to analyze significance levels. Specific significance levels are found in figure legends. $P < 0.05$ was considered significant.

Results and Discussion
Oncogenic KRAS phosphorylation at Ser-181 is required for tumor growth
To test the prediction that phosphorylation at Ser181 of oncogenic KRAS was required to support tumor growth, NIH3T3 stable cell lines expressing similar levels of oncogenic HA-tagged G12V KRAS, namely HA-KRAS-G12V-S181 (S181), non-phosphorylatable HA-KRAS-G12V-S181A (S181A), or phosphomimetic HA-KRAS-G12V-S181D (S181D) (Fig. 1A) were subcutaneously injected into nude mice and tumor growth was monitored over time. Tumor formation was nearly abolished in cells expressing non-phosphorylatable S181A (Fig. 1B and C and Supplementary Tables S1 and S2). Furthermore, a dramatic increase in tumor growth was observed for phosphomimetic S181D mutant compared with the phosphorylatable S181. No tumor growth was observed...
when injecting NIH3T3 cells stably expressing wild-type HA-KRAS (Supplementary Table S1), which confirmed that both engraftment and growth was driven by our oncogenic KRAS phosphomutants. Interestingly, in spite of the dramatic diminished growth of non-phosphorylatable S181A-derived tumors, KRAS oncprotein was overexpressed in those tumors compared with the S181- or S181D-derived tumors (Fig. 1D). This suggests that, during the process of tumor development, cells with higher expression of non-phosphorylatable KRAS are positively selected in an attempt to overcome the lower tumorigenic activity exhibited by this mutant. Similar results were obtained when injecting in nude mice two independently immortalized S181A clones with distinct expression levels. Again in S181A clones, tumor growth was highly compromised irrespectively of the KRAS protein expression level (Supplementary Fig. S1).

The impaired tumor growth of the non-phosphorylatable S181 G12V mutant associated with a distinct histologic pattern. S181A tumors were composed mostly by cells with an epithelioid appearance and with a significant lymphocytic infiltration (S181A 10.00 ± 1.08 lymphocytes per × 100 field vs. S181 2.25 ± 0.63 vs. S181D 1.25 ± 0.25; P < 0.0001; Fig. 3 and Supplementary Fig. S3). Of note, the same histology has been previously reported for sarcomas harboring the mild KRAS codon 13 mutations (16). In contrast, S181- and S181D-derived sarcomas were composed of a fusocellular population showing a hemangiopericytoid pattern. The non-phosphorylatable S181A tumors had also a lower mitotic rate (S181A 4.00 ± 1.53 mitotic cells per × 100 field vs. 27.00 ± 4.12 for S181 vs. 54.75 ± 4.99 for S181D; P < 0.0001) and were the only tumors showing detectable levels of cleaved caspase-3, a bona fide apoptosis marker (Fig. 1D and Supplementary Fig. S2A), together with a significant increase in TUNEL-positive cells (Fig. 2A and B). This is in accordance with the decreased resistance to apoptosis already reported in vitro for S181A compared with S181 and S181D oncogenic KRAS mutants (14). In agreement with the prediction of a stronger activity of KRAS upon phosphorylation, the phosphomimetic S181D-derived tumors exhibited higher ERK and AKT activity (Fig. 1D), accompanied by a pronounced increase in the number of positive cells for the proliferative marker Ki-67 (Fig. 2A and B). Although mild increase in TUNEL-positive cells was also observed in S181D compared with S181 tumors, S181A tumors were the ones exhibiting the highest degree of apoptosis. Intriguingly, S181A tumors showed higher cyclin B1 expression than the others (Fig. 1D) in line with the requirement of increased cyclin B1 for apoptosis induction previously reported in several tumor cell lines (17–19). Moreover, cyclin B1 overexpression has already been related to the mild transforming phenotype of codon 13 KRAS mutations in NIH3T3 models (16).

Thus, the impossibility of phosphorylating oncogenic KRAS dramatically changes growth pattern rendering activating mutations much less aggressive and demonstrating the relevance of this posttranslational modification in KRAS-driven transformation.

PKC inhibitors diminish oncogenic KRAS-mediated tumor growth

The dependence of oncogenic KRAS on S181 phosphorylation makes oncogenic KRAS a putative target for protein kinase...
Figure 4. Pharmacologic inhibition of PKC activity inhibits tumor growth and KRAS-G12V–dependent signaling pathways in a K-RasG12V Ser181-phosphorylation dependent manner. NIH3T3 cells stably expressing either HA-KRAS-G12V (S181) or HA-KRAS-G12V-S181D (S181D) were injected into each flank of nude mice. When tumor reached a designated volume of approximately 150 mm³ (latency time shorter for S181D tumors), animals were divided into two groups (each group n = 10) and treated daily either with vehicle (5% DMSO) or Bryostatin-1 (Bryo; 75 µg/kg) for 6 days, and euthanized the next day. A, Western blot analysis showing HA-KRAS expression in different pools of NIH3T3 cells the day of injection. Injected NIH3T3 pools (arrows) were chosen among the ones with equivalent expression for HA-KRAS-G12V (S181) or HA-KRAS-G12V-S181D (S181D). B, Increment in tumor size was obtained by comparing tumor volume at the starting day (day 1) and at day 7 of treatment. Dissected tumors from the nude mice are displayed below the graph. Scale bar, 25 mm. C, total cell lysates of representative excised tumors were immunoblotted to detect the indicated proteins (numbers indicate different tumors). Anti-GAP120 was used as loading control. D, Quantifications of Ki-67 labeling (left) and TUNEL labeling (right) were made from at least two different tumors per mutant (each point represents a counted field). * * *, P < 0.0001; **, P < 0.001; * P < 0.01; P value for Student two-tailed t test; ns, nonsignificant differences; mean and SEM are represented. E, cellular extract from tumors were resolved in Phos-Tag SDS–PAGE gels and immunoblot was performed using anti-HA antibody. An aliquot of a S181 tumor from an animal treated with DMSO was incubated, before electrophoresis, at 30°C with phosphatase λ (λ) or only with buffer (Ctl) to discard unspecific effects due to heating samples.
inhibitors. Since PKCs are considered to be the putative kinases for KRAS Ser181 phosphorylation (13, 20, 21), we tested whether treatment with two general PKC inhibitors that are clinically relevant (Bryostatin-1 and Edelfosine; refs. 22–26) were able to revert tumor growth in a dephosphorylation-dependent manner.

Bryostatin-1 inhibits PKC activity when administrated in vitro at concentrations as low as 0.1 nmol/L (22). In our experiments, we used 75 μg/kg, a dose that was previously used for in vivo PKC inhibition (27). As shown in Fig. 4B, Bryostatin-1 treatment significantly reduced tumor growth of S181, whereas no effect was evident on “non-dephosphorylatable” S181D tumors. Of note, we found that Bryostatin-1 treatment, in accordance to its general PKC inhibitor activity, efficiently downregulated both total and active PKCδ levels as previously described (Fig. 4C; ref. 21). Tumor reduction with Bryostatin-1 treatment was associated with a decreased ERK activity that was specific for S181 phosphorylatable mutant. Moreover, apoptosis was induced as shown by an increase of cleaved caspase-3 levels and TUNEL-positive cells (Fig. 4C and D and Fig. S2B). Concomitantly, cell proliferation was inhibited (Fig. 4D); whereas cyclin B1 expression was increased (Fig. 4C). In this way, Bryostatin-1 treatment showed high specificity for the dephosphorylatable S181 tumors and interestingly, treatment of these tumors efficiently recapitulated the growth and signaling pattern of S181A tumors (22) shown in Fig. 1. Accordingly, PKC inhibition did not affect growing and signaling pattern, nor increased apoptosis in the non-dephosphorylatable KRAS S181D tumors.

To further confirm the striking results obtained with Bryostatin-1 treatment on mice, we treated the same stable transfected NIH3T3 mice grafts with Edelfosine. This is an ether lipid analog to HMG with reported strong PKC inhibitor activity both in vitro (28, 29) and in vivo (30). As shown in Supplementary Fig. S4, we reproduced a significantly reduced tumor growth of S181 and again no significant effect was observed in “non-dephosphorylatable” S181D tumors.

Altogether, these results suggested that both Bryostatin-1 and Edelfosine, by blocking PKC activity, impair tumor growth inducing KRAS dephosphorylation and subsequent apoptosis. To formally prove this hypothesis, detection of KRAS phosphorylation was necessary. Since no suitable antibodies are available, we used the Phos-Tag-based approach (31, 32) to determine the oncogenic KRAS phosphorylation status in the generated tumors. This method is based on the fact that a complex formation between the phosphate group of a phosphorylated protein and a divalent metal ion in Phos-Tag reduces the mobility of the phospho-protein during the electrophoresis separation, thus allowing resolution of phosphorylated and non-phosphorylated proteins into different bands. As shown in Fig. 4E, a slow migrating band of HA-KRAS could...
be observed in the tumors generated from cells expressing the S181 oncogenic KRAS that was absent in S181D tumors. Disappearance of this band upon λPhosphatase treatment corroborated it was phosphorylated KRAS. Most interestingly, in Bryostatin-1- and Edelfosine–treated animals, phosphorylated KRAS was no longer observed (Fig. 4E and Supplementary Fig. S4). Together, these observations reinforce the notion that PKC-dependent Ser181-phosphorylation of oncogenic KRAS is required for tumorigenesis. This effect may account for the previously reported inhibition of different KRAS-driven tumor xenografts by PKC pharmacologic inhibition (30, 33, 34). Interestingly, it has also been shown that PKCδ knockdown prevents apoptosis and promotes tumorigenesis in cells addicted to aberrant KRAS signaling (35–37).

**Human cell lines require phosphorylation of KRAS for survival and tumor growth**

To determine whether the requirement for KRAS phosphorylation observed in our NIH3T3 KRAS transformation model was also involved in human cell line tumorigenesis, we ectopically expressed the HA-KRAS-G12V phosphomutants described above in the human colorectal cancer cell line DLD-1 but previously knocked out for the oncogenic endogenous KRAS allele (DLD1<KRASwt/>).

We found that under serum-saturating growth conditions (10% FCS), human colon cancer cells DLD-1 expressing S181A mutant exhibited a significantly reduced growth compared with phosphomimetic S181D-expressing cells (Fig. 5A). Trying to recapitulate tumor growth conditions, we evaluated cell growth under serum-limiting conditions (0.1% FCS). After 4 days of starvation, cells stably expressing S181A showed significantly higher reduced growth under serum starvation culture conditions compared with S181 and phosphomimetic S181D (Fig. 5A). Moreover, S181A exhibited increased sensitivity to apoptosis under serum deprivation or by adriamycin-induced genotoxic damage (Fig. 5A and B), thus demonstrating a proapoptotic effect of the S181A oncogenic KRAS.

To evaluate real tumorigenic capacity of these cells, subcutaneous injection of DLD1<KRASwt/> expressing either HA-KRAS-G12V or HA-KRAS-G12V-S181A phosphomutants was performed. S181A-derived tumors were significantly smaller than S181 tumors (Fig. 5C). This confirmed the requirement of KRAS S181 phosphorylation for tumorigenesis of human colon cell lines.
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was previously incubated with phosphatase immunoblotting using anti-KRAS antibodies. An aliquot of each extract treatment, it was shown that at doses between 1 μmol/L and 20 μmol/L for BIM and 1.5 μmol/L and 10 μmol/L for G66983, cells expressing oncogenic KRAS with S181 exhibited significantly enhanced sensitivity to PKC inhibition compared with the phosphomimetic non-dephosphorylatable mutant (Fig. 6A).

Most importantly, after PKC inhibition, S181 cells lost its KRAS phosphorylation as shown by Phos-tag SDS–PAGE gels (Fig. 6A).

Finally, we evaluated the ability of a set of PKC inhibitors to reduce proliferation together with KRAS phosphorylation in a panel of human cell lines from different origin harboring oncogenic KRAS. We found that at doses reported to inhibit PKC (22, 29, 39, 41), cell growth was compromised. Most importantly, after 12 hours of treatment, band corresponding to phospho-KRAS was lost, thus reinforcing the idea that PKC inhibition is able to revert growth in a KRAS S181-dependent manner (Fig. 6B).

Figure 7. Detection of the phosphorylated form of oncogenic KRAS in human pancreatic ductal adenocarcinomas. Extract from 5 different human pancreatic ductal adenocarcinomas with oncogenic mutations in codon 12 of KRAS (#1 G12D heterozygous; #2 G12D heterozygous; #3 G12D homozygous; #4 G12V heterozygous; #5 G12V heterozygous), were resolved in Phos-Tag SDS–PAGE or SDS–PAGE followed by immunoblotting using anti-KRAS antibodies. An aliquot of each extract was previously incubated with phosphatase λ. Anti-GAP120 was used as loading control.

A preferential activation of endogenous wild-type H- and X-RAS alleles induced by the oncogenic KRAS has recently been reported (38). To check whether diminished growth capacity of S181A was due to lack of activation of the endogenous RAS isoforms, RBD pull-down assays were performed to test GTP loading of endogenous RAS isoforms. Lower GTP loading of endogenous Ras in the S181A-expressing cells was not observed compared with the other phosphomutants (Supplementary Fig. S5).

Next, we investigated whether the S181 phosphorylation observed in our model system was also present in human tumors. To do so, a set of orthotopic xenografts derived from carcinomas of the exocrine pancreas were analyzed. Five tumors harboring codon 12 KRAS mutations were tested. As shown in Fig. 7, by using Phos-Tag SDS–PAGE, several bands were detected in all tumors using the anti-KRAS antibody. Treatment with λ phosphatase (Fig. 7) and two-dimensional electrophoresis analysis corroborated the presence of phosphorylated KRAS in these human tumors (Supplementary Fig. S6). Thus, the presence of phospho-KRAS in human malignancies emphasizes the alleged requirement of this modification for human KRAS-driven tumorigenesis.

Altogether, the results depict a scenario of a novel tight regulation of KRAS oncogenicity by phosphorylation at S181. We have recently shown that although phosphorylated KRAS is mainly found at the plasma membrane (41, 42), phosphorylated K-Ras forms distinct plasma membrane signaling platforms that induce preferential activation of main KRAS effectors involved in oncogenesis. Interestingly, this distinct functionality could be reverted by PKC inhibitors (41). This would give a rationale for the strikingly different tumorigenic activity of oncogenic KRAS according to its S181 phosphorylation status.

The fact that, as we show, this could be efficiently pharmacologically inhibited raises the possibility of novel therapeutic strategies targeting KRAS-driven human malignancies. So far, clinical trials with PKC regulators have been disappointing mostly because of the lack of selectivity and unacceptable toxicity (40). The identification of KRAS as a key PKC target may help in developing specific inhibitors of KRAS phosphorylation.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
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