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STK33 Kinase Activity Is Nonessential in KRAS-Dependent Cancer Cells

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Abstract

Despite the prevalence of KRAS mutations in human cancers, there remain no targeted therapies for treatment. The serine–threonine kinase STK33 has been proposed to be required for the survival of mutant KRAS-dependent cell lines, suggesting that small molecule kinase inhibitors of STK33 may be useful to treat KRAS-dependent tumors. In this study, we investigated the role of STK33 in mutant KRAS human cancer cells using RNA interference, dominant mutant overexpression, and small molecule inhibitors. As expected, KRAS downregulation decreased the survival of KRAS-dependent cells. In contrast, STK33 downregulation or dominant mutant overexpression had no effect on KRAS signaling or survival of these cells. Similarly, a synthetic lethal siRNA screen conducted in a broad panel of KRAS wild-type or mutant cells identified KRAS but not STK33 as essential for survival. We also obtained similar negative results using small molecule inhibitors of the STK33 kinase identified by high-throughput screening. Taken together, our findings refute earlier proposals that STK33 inhibition may be a useful therapeutic approach to target human KRAS mutant tumors. *Cancer Res; 71(17); 5818–26. ©2011 AACR.*

Introduction

KRAS activating mutations are present in approximately 30% of human cancers, making KRAS one of the most frequently mutated oncogenes. Multiple preclinical studies *in vitro* and *in vivo* have shown that blocking the activity of mutant KRAS is an effective way to inhibit tumor growth, suggesting that drugs that block this pathway could be effective anticancer therapies (1). Strategies to target KRAS directly, such as downregulating its expression or disrupting its membrane localization through farnesyltransferase inhibitors or geranylgeranyl transferase inhibitors, have not yet been successful in the clinic (2). Multiple attempts to target KRAS indirectly by blocking its effectors have been undertaken and some are still being tested. For example, potent and selective inhibitors of MAP/ERK kinase 1/2 (MEK1/2) are currently being tested in several clinical trials (www.clinicaltrials.gov). Although some of these efforts seem promising, a more complete understanding of the complex and extensive network of KRAS effectors and regulators may provide alternative and more efficient therapeutics. Several investigators have sought to directly identify critical effectors of KRAS through genetic manipulations. For example, screening for synthetic lethal interactions in the context of KRAS mutations could in principle lead to the identification of novel genes critical for KRAS activity. A number of studies have been published recently describing such screens and potential drug targets. In one of these studies, STK33, a poorly characterized kinase, was identified as a candidate drug target for mutant KRAS tumors (3). STK33 is a serine–threonine kinase that belongs to the calcium/calmodulin–dependent family of kinases (4). The physiologic function of STK33 is unknown and STK33 does not seem to be genetically altered in cancers (3). However, downregulation of STK33 by RNA interference (RNAi) or inhibition of its kinase activity by overexpression of a kinase dead (KD) K145M STK33 protein conferred a lethal phenotype to mutant KRAS cell lines (3). Suppression of STK33 expression by either method resulted in the inhibition of p70S6K and RPS6 phosphorylation but had no effect on the phosphorylation of AKT, ERK1/2, or mTOR, suggesting that STK33 is downstream from these nodes, but upstream from p70S6K.

Considering the attractiveness of targeting a kinase to treat a well-defined patient population (KRAS mutated tumors), we sought to confirm the findings in Scholl and colleagues (3), and, in parallel, to identify small molecule inhibitors of STK33 kinase activity to further probe STK33 as a potential therapeutic target. However, our results show that neither

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downregulation of STK33 by RNAi nor overexpression of K145M STK33 affected the viability of mutant KRAS-dependent cell lines. Small molecule STK33 kinase inhibitors identified by high throughput screening did not inhibit cell viability in a mutant KRAS-dependent manner. Together our data suggest that STK33 inhibition does not block the growth of KRAS-dependent cell lines.

Materials and Methods

Cell lines and reagents

Cell lines were obtained from American Type Culture Collection, the European Collection of Cell Cultures, or from the German Resource Centre for Biological Material (DSMZ, Germany) and were cultured for less than 6 months. We have confirmed the KRAS status by sequencing in the following cell lines: U937 (KRAS wild-type), PANC-1 (KRAS G12D), DLD-1 (KRAS G13D), NB4 (KRAS A18D), NOMO-1 (KRAS G13D), OCI-AML3 (wild-type), and SKM-1 (KRAS K117N). The KRAS status for all other cell lines was obtained from the Wellcome Trust Sanger Institute Cancer Genome Project website. All cell lines were cultured as recommended by the respective vendors and all cell assays were done in serum-containing growth media unless otherwise specified. Antibodies for Western blotting were obtained from Abnova (STK33 clone 1F10), Santa Cruz Biotechnology (KRAS, Actin), and Cell Signaling Technology [P-p70S6K (T389), P-RPS6 (S235/236), and FLAG]. The siRNA oligonucleotides were either commercially available or custom synthesized and are described in Supplementary Methods. Commercial sources of full-length, active STK33 proteins were from Millipore (catalog no. num; 14-671) and Invitrogen (catalog no. PV4343). Full-length STK33 was also produced at Amgen Inc. (see Supplementary Methods). The LANCE *Ultra* Kinaselect Ser/Thr kit (PerkinElmer) was used to identify STK33 substrates.

RNAi and overexpression of STK33

NOMO-1 and SKM-1 cells were transfected with siRNA using Transductin (Integrated DNA Technologies, Inc.), a peptide-based delivery system. Cells were incubated with peptide-siRNA complexes for 24 hours in growth media containing glycosaminoglycan-depleted FBS. Following incubation, cells were washed in growth media and replated (day 1) for Western blotting and viability assays as described below. HCT-116 and MDA-MB-231 cells were transfected with siRNA in duplicate 96-well plates using RNAiMAX (Life Technologies) with a final siRNA concentration of 30 nmol/L. RNA expression levels were quantified from one of the two 96-well plates 24 hours after transfection using the QuantiGene branched DNA assay. Viability was quantified from the other 96-well plate 96 hours after transfection using CellTiterGlo (Promega) according to the manufacturer's protocol.

For DNA transfections, PANC-1 and DLD-1 cells were plated at 10,000 cells per well in 96-well plates for viability assays or 300,000 cells per well in 6-well plates for Western blotting. The following day, cells were transfected (day 0) with pcDNA3.1-FLAG STK33 or pcDNA3.1-FLAG K145M STK33 or with the empty vector as a control using FuGeneHD (Roche) according to the manufacturer's protocol. For Western blotting, siRNA or DNA transfected cells were incubated in serum-free media [Dulbecco's modified Eagle's medium/0.1% bovine serum albumin (BSA)] for 4 hours before harvest on day 3 after transfection.

Quantification of RNA expression levels

RNA expression levels were determined using the QuantiGene branched DNA (bDNA) assay (Affymetrix) following the manufacturer's instructions. The probe sets to detect STK33, KRAS, and cyclophilin B RNA are described in Supplementary Methods. Assays were carried out using cell lysates prepared with 1X Quantigene Lysis Mixture (Affymetrix).

High throughput siRNA screens

High throughput screens of siRNA libraries were done in 27 solid tumor cell lines, including cell lines mutant for KRAS. A cell viability assay using CellTiterGlo was done 96 or 120 hours posttransfection. Detailed description of experimental procedures and statistics are found in the Supplementary Methods.

Kinase assays and small molecule library screen

Immunoprecipitation kinase assay. On day 3 posttransfection, DLD-1 cells were lysed on ice in Triton lysis buffer containing fresh protease and phosphate inhibitors. FLAG-STK33 was immunoprecipitated overnight at 4°C in lysis buffer using an anti-FLAG antibody prebound to Protein A/G-Plus agarose beads (Santa Cruz Biotechnology). After overnight incubation, the beads were washed extensively in Triton lysis buffer, then with kinase buffer (50 mmol/L HEPES pH 7.4, 15 mmol/L $MgCl_2$, 1 mmol/L DTT, 0.02% BSA). Following the washes, a mixture of 50 μCi ^{33}P γ-ATP (PerkinElmer) and 10 μmol/L cold ATP was added to each IP to initiate the autophosphorylation reaction. After 90 minutes at 30°C the kinase reactions were stopped by the addition of SDS sample buffer and the samples were run on 10% Tris/glycine gels, fixed, and exposed to X-Ray film (Kodak).

To identify STK33 substrates, STK33 proteins from Millipore or Invitrogen were used with the LANCE *Ultra* Kinaselect Ser/Thr kit in a lanthanide chelate excite (LANCE) time-resolved fluorescence resonance energy transfer (TR-FRET) kinase assay. The kinase buffer contained 50 mmol/L HEPES pH 7.0, 10 mmol/L $MgCl_2$, 1 mmol/L EGTA, 2 mmol/L DTT, 0.01% Tween 20, 0.05% BSA, 20 nmol/L STK33, and 200 μmol/L ATP as suggested by the manufacturer. STK33, substrate, and buffer were mixed and the kinase reaction was carried out at room temperature for 90 minutes. For detection of the phosphorylation signal, each reaction mix was incubated for 2 hours with the appropriate Eu-anti-phospho-peptide antibody. The LANCE signals were detected on a Rubystar instrument (BMG Labtech Inc.).

A high-throughput screen to identify STK33 inhibitors was conducted using an optimized kinase assay (Km ATP 20 μmol/L) against a small molecule library containing 410,432 compounds. The compounds were tested at a concentration of 15 μmol/L. The kinase reaction buffer contained 50 mmol/L HEPES pH 7.0, 15 mmol/L $MgCl_2$, 2 mmol/L DTT, 0.01% Tween 20, 0.05% BSA, 5 nmol/L STK33, 20 μmol/L ATP, and 50 μmol/L ULight-p70S6K

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(T389) as a substrate (PerkinElmer). The kinase reaction was at room temperature for 3 hours. The detection buffer contained 125 pmol/L Eu-anti-p-p70S6K and 15 mmol/L EDTA, and the reaction was carried out at room temperature for 2 hours. The fluorescence signals were read on an EnVision plate reader instrument (PerkinElmer). Follow-on 22-point dose–response assays were used to determine the IC_{50} for the 1,043 most potent STK33 inhibitors.

Cell-based assays

Cell viability assays. Following transfection, NOMO-1 and SKM-1 cells were plated in growth media (day 1) at 40,000 cells per well in black-walled 96-well plates (Corning) for viability assays. To test the effects of the STK33 kinase inhibitors on cell viability, NB4, SKM-1, U937, and OCL-AML3 cells were plated at 2,000 cells per well in 96-well plates in growth media with increasing concentrations of STK33 inhibitors. Viability was assayed at specified time points using ATPlite (PerkinElmer) according to the manufacturer's protocol. Luminescence was measured using a SpectraMax plate reader (Molecular Devices). Excel XLFit4 was used to determine the IC_{50} of the small molecules.

p-RPS6 assay. Cells were grown in their respective serum-containing growth media until the day of the experiment. On the day of the experiment, cells were washed and resuspended in OptiMEM + 25 mmol/L HEPES, and exposed to increasing concentrations of small molecules for 1 hour at 37°C. For the detection of the intracellular RPS6 phosphorylation, the AlphaScreen SureFire cellular kinase assay kit (p-RPS6 S240/S244) and AlphaScreen protein A kit (PerkinElmer) were used. The detection mix was composed of 40 parts reaction buffer, 10 parts activation buffer, 1 part acceptor bead, 1 part donor bead, and 20 parts lysis buffer.

Results

STK33 knockdown by RNAi has no effect on the viability of mutant KRAS-dependent cell lines.

To test whether STK33 has a synthetic lethal relationship with mutant KRAS, we transiently transfected KRAS wild-type or mutant cells lines with siRNAs directed at STK33 or KRAS. Our experiments were focused on acute myelogenous leukemia (AML) cell lines, which were extensively used to characterize the synthetic lethal interaction between STK33 and mutant KRAS (3). NOMO-1 and SKM-1 cells were transfected with control siRNAs (scrambled-scram, cyclophilin B–cycl B, luciferase-lucif) or with siRNAs against STK33 or KRAS. Messenger RNA and protein levels were analyzed 24 hours or 3 days after transfection, respectively. Downregulation of KRAS expression was accompanied by a reduction of p70S6K and RPS6 phosphorylation in both cell lines (Fig. 1A and C). However, although downregulation of STK33 was

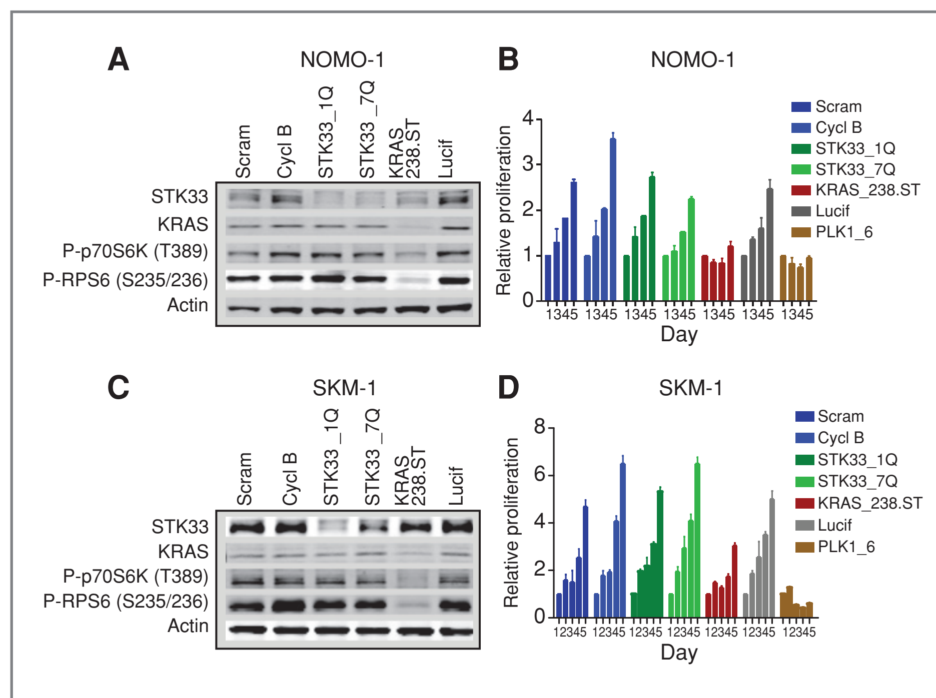

Figure 1. STK33 knockdown by RNA interference has no effect on the viability of mutant KRAS-dependent cell lines. A, NOMO-1(KRAS G13D) cells were transiently transfected with control siRNA or siRNA for STK33 or KRAS. SiRNA to PLK1 was used as a positive control. A fraction of the transfected cells was used to generate cell lysates for Western blotting. STK33 and KRAS protein levels, as well as p70S6K and RPS6 phosphorylation, were monitored. B, cell viability was monitored periodically for 5 days and expressed as relative proliferation (fold over value on day 1, the time of replating following cell transfection). The experiment was repeated at least 3 times with similar results. One representative experiment is shown. Error bars represent the SD of each assay condition done in triplicate. C, same as A, but with SKM-1 (KRAS K117N) cells. D, same as B, but with SKM-1 cells. A–D are representative examples; each experiment was repeated 3 times with similar results.

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achieved (Fig. 1A and C and Supplementary Fig. S1), no change in the phosphorylation of p70S6K or RPS6 was detected (Fig. 1A and C).

In parallel, cell viability was measured for 5 days following transfection. Although KRAS knockdown in NOMO-1 and SKM-1 cells was consistently associated with a decrease in cell viability, STK33 knockdown had no detectable effect on the viability of these cell lines (Fig. 1B and D). Our data therefore suggest that downregulation of STK33 does not impede the survival of the KRAS-dependent NOMO-1 and SKM-1 cell lines.

To extend these observations, high-throughput screens of siRNA libraries were done in 27 solid tumor cell lines, including cell lines mutant for KRAS. SiRNAs directed at KRAS significantly inhibited ($P < 0.05$) the viability of mutant KRAS cell lines. However, siRNAs directed at STK33 showed no significant effect on cell viability in any of the tested cell lines, irrespective of their KRAS status (Fig. 2A and B). Knockdown of STK33 and KRAS RNA with the siRNAs used in the high-throughput screens was verified in 2 KRAS mutant cell lines (Supplementary Figs. S2 and S3). Our data confirmed the efficient knockdown of both genes, but also uncovered off-target effects for 2 siRNAs, STK33_2Q and STK33_7Q, which inhibited cell viability (Supplementary Figs. S2 and S3).

Overexpression of the kinase dead K145M STK33 has no effect on the viability of mutant KRAS-dependent cell lines

Another approach to study, the role of STK33 kinase activity in mutant KRAS cell lines, consisted of overexpressing a kinase dead version of STK33 in which the catalytic lysine is mutated to a methione (K145M STK33). DLD-1 and PANC-1 cells were chosen for these experiments because they carry a KRAS mutation (G13D and G12D, respectively) and have been reported to be dependent on KRAS and STK33 for proliferation and survival (3). STK33 protein levels are higher in DLD-1 cells compared with PANC-1 cells, but both cell lines have similar levels of p70S6K and RPS6 phosphorylation (Supplementary Fig. S4). In general, STK33 RNA and protein levels are variable across cell lines and protein levels do not correlate with the levels of p70S6K and RPS6 phosphorylation (Supplementary Fig. S4A and B).

To confirm that K145M STK33 has no catalytic activity, FLAG-tagged wild-type or K145M STK33 were overexpressed in DLD-1 cells by transient transfection, immunoprecipitated, and incubated with ^{33}P γ-ATP. Weak autophosphorylation of wild-type STK33 was observed following its overexpression, consistent with published data (3, 5), but no autophosphorylation of K145M STK33 was observed (Fig. 3A). Endogenous STK33 kinase activity was low in several cell lines tested, suggesting that it is a weak kinase in cells (data not shown). We also tested the purified recombinant K145M STK33 protein in a kinase assay with the p70S6K peptide as a substrate. K145M STK33 was again confirmed to be inactive (Fig. 4B, right). We then monitored the effect of overexpression of wild-type or K145M STK33 on the levels of p70S6K and RPS6 phosphorylation and on the viability of DLD-1 and PANC-1 cell lines. Overexpression of wild-type STK33 had no effect on the levels of p70S6K or RPS6 phosphorylation or on the

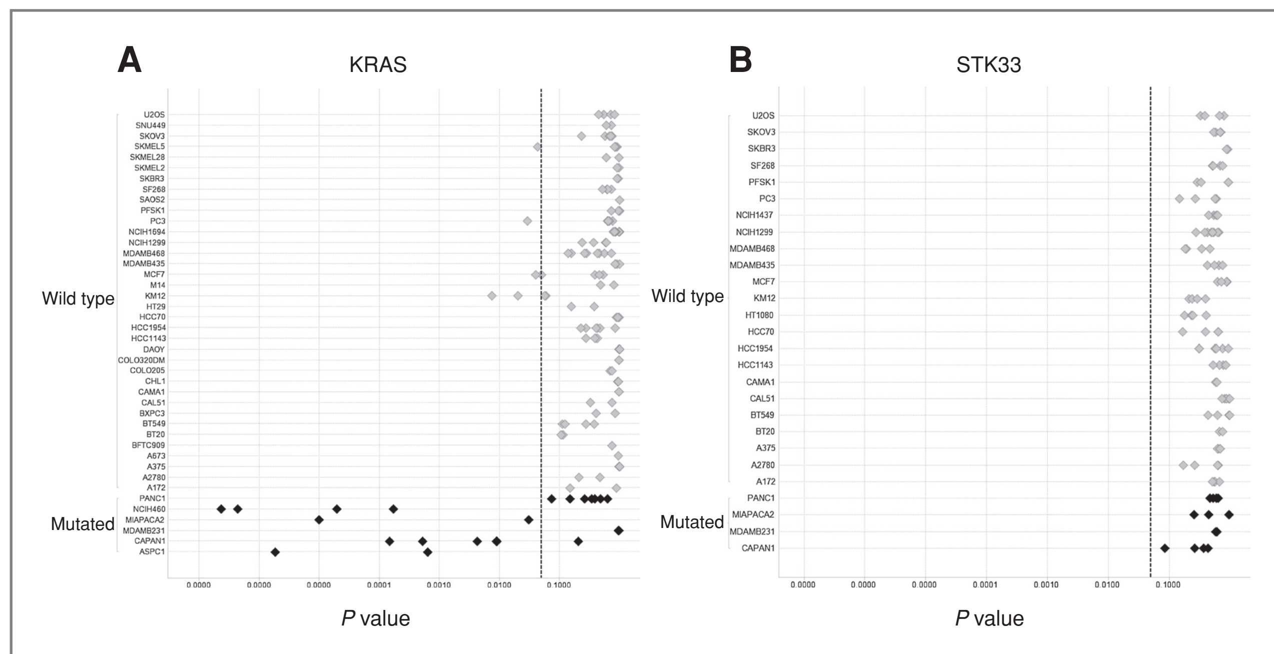

Figure 2. Mutant KRAS cell lines are sensitive to knockdown of KRAS, but not to knockdown of STK33. A and B, multiple cancer cell lines were transfected in a high-throughput format with libraries of siRNA containing multiple triggers for each gene. Cell viability determined 96 or 120 hours after transfection of siRNA was expressed as a *P* value for KRAS (A) or STK33 (B). Each diamond represents the result of one siRNA screen. Most cell lines were tested several times using different transfection conditions. Diamonds to the left of the dashed line ($P < 0.05$) indicate a significant effect of gene knockdown on cell viability in a given experiment. Black diamonds are results for KRAS mutant cell lines, and gray diamonds are for KRAS wild-type cell lines.

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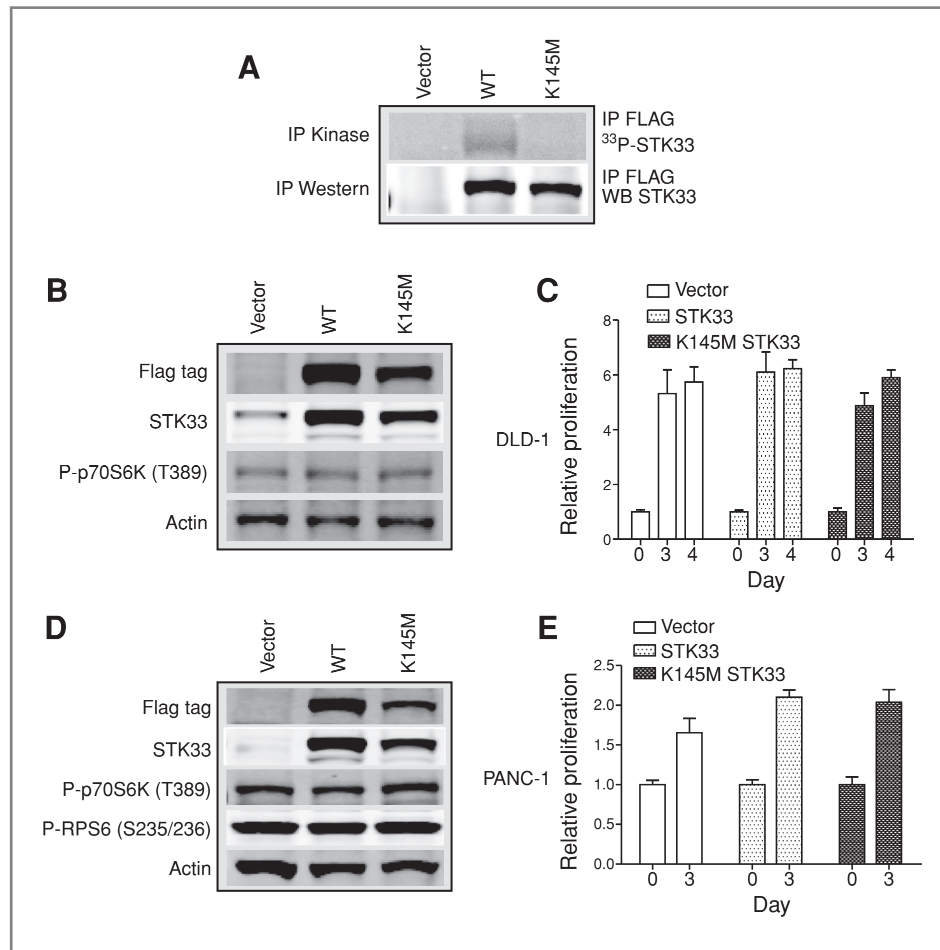

Figure 3. Overexpression of K145M STK33 has no effect on the proliferation of mutant KRAS-dependent cell lines. A, DLD-1 (KRAS G13D) cells were transiently transfected with a control vector or with a vector expressing wild-type (WT) or K145M STK33 cDNA with a FLAG tag. STK33 was immunoprecipitated (IP) with an antibody against the FLAG tag and incubated with ^{33}P γ-ATP. ^{33}P incorporation in STK33 is detectable, but weak, only in the presence of wild-type STK33. An aliquot of each immunoprecipitate was run on a Western blot and probed with an antibody against STK33. B, DLD-1 cells were transiently transfected as in A. Cell lysates were used to monitor STK33 levels and p70S6K phosphorylation at day 3. C, same as B, except that cell viability was assayed on days 3 and 4 following transfection. The data are expressed as relative proliferation (fold over value on day 0, which was the time of cell transfection). The experiment was repeated at least 3 times with similar results. One representative experiment is shown. Error bars represent the SD of each assay condition done in triplicate. D, same as B, except in PANC-1 (KRAS G12D) cells. E, same as C, but with PANC-1 cells.

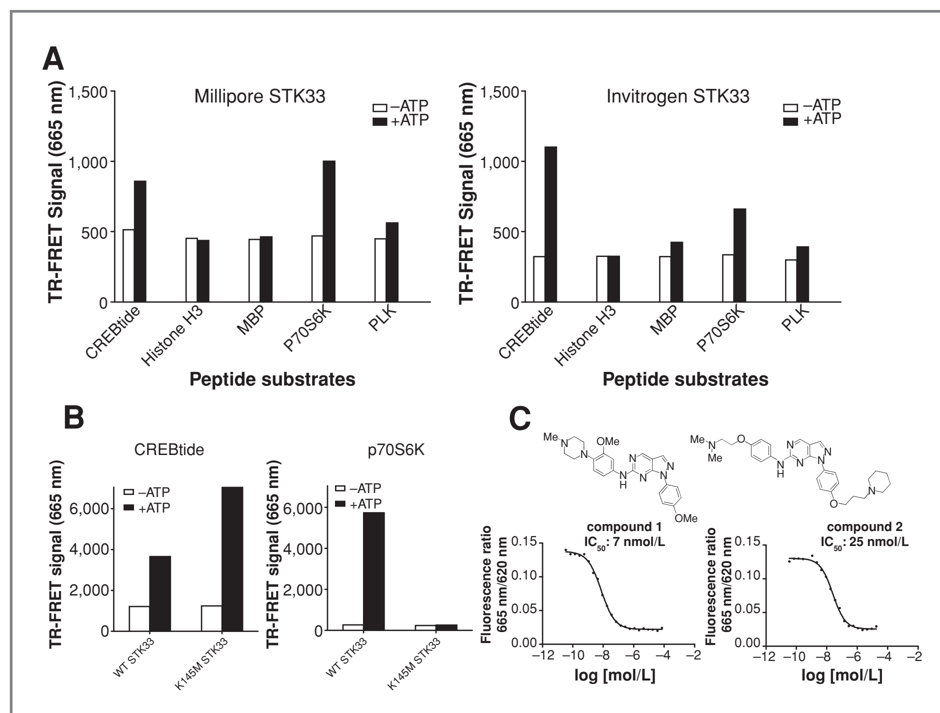

Figure 4. Identification of STK33 small molecule kinase inhibitors. A, full-length STK33 proteins from either Millipore (left) or Invitrogen (right) were used to test the ability of STK33 to phosphorylate 5 different peptides using generic kinase assay conditions as described in Materials and Methods. The *y*-axis illustrates TR-FRET signal (absolute counts for energy emission detected at 665 nm after energy transfer). Only the CREBtide and p70S6K peptides were phosphorylated by STK33 in the presence of ATP. B, kinase assays were done with a full-length WT or K145M STK33 protein expressed and purified at Amgen Inc. with the CREBtide peptide (left) or with the p70S6K peptide (right) as substrates. One of multiple representative experiments is shown in A and B. C, examples of IC_{50} curves for 2 potent STK33 inhibitors.

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Table 1. High-throughput screen for STK33 kinase inhibitors

	Number of compounds tested	Number of hits (% hit rate)
Screen	410,432	3,862 (0.95%)
Confirmation	3,862	2,727 (71%)
Dose–response	1,043	1,000
Most potent hits	6 compounds < 10 nmol/L, 69 compounds < 100 nmol/L, 443 compounds < 1 μmol/L, 358 compounds < 3 μmol/L, 124 compounds < 10 μmol/L	

NOTE: In the initial screen, all molecules were tested at a concentration of 15 μmol/L. The kinase assay was done using a 22-point dilution of the 1,043 most potent compounds to determine their respective IC_{50}s.

viability of DLD-1 or PANC-1 cells (Fig. 3B–E). Similarly, overexpression of the K145M STK33 in these 2 cell lines had no effect on either of these parameters (Fig. 3B–E).

Identification of small molecule STK33 kinase inhibitors

To directly address whether the STK33 kinase activity is important for the survival of mutant KRAS cell lines, we developed a high-throughput STK33 kinase assay to screen for potent small molecule inhibitors. Because little is known about the potential substrates for STK33 (5), a small commercial set of substrates, including peptides for CREB (CREBtide), p70S6K, polo-like kinase (PLK), histone H3, and myelin basic protein, was screened using full-length STK33 proteins from Millipore or Invitrogen. These preliminary experiments showed that the CREBtide and p70S6K peptides were potential substrates for the 2 separate sources of STK33 (Fig. 4A). Full-length STK33 was then expressed and purified by us in sufficient quantities to characterize its enzyme kinetic parameters, to optimize kinase assay conditions, and to conduct a high-throughput small molecule screen. During assay optimization, we found that the CREBtide peptide was phosphorylated in an STK33-independent fashion (Fig. 4B, left; and data not shown). Unexpectedly, CREBtide phosphorylation was detected after a kinase reaction in the presence of K145M STK33, suggesting the presence of some contaminant kinase in this preparation. On the other hand, the phosphorylation of the p70S6K peptide was closely linked to the levels of STK33 protein purity and was absent from the K145M STK33 preparation (Fig. 4B, right; and data not shown) suggesting that p70S6K is a more selective substrate. We therefore screened a small molecule library using an STK33 kinase assay with a p70S6K peptide as a substrate. More than 400,000 small molecules were screened at a concentration of 15 μmol/L. The hit rate was 0.95% and the confirmation rate was 71%. The compound concentrations that inhibited the kinase activity by 50% (IC_{50}) were determined for the 1,043 most potent hits. We identified 6 molecules that inhibited STK33 kinase activity with IC_{50}s less than 10 nmol/L and 69 molecules with IC_{50}s less than 100 nmol/ (Table 1). Examples of IC_{50} curves are shown in Fig. 4C.

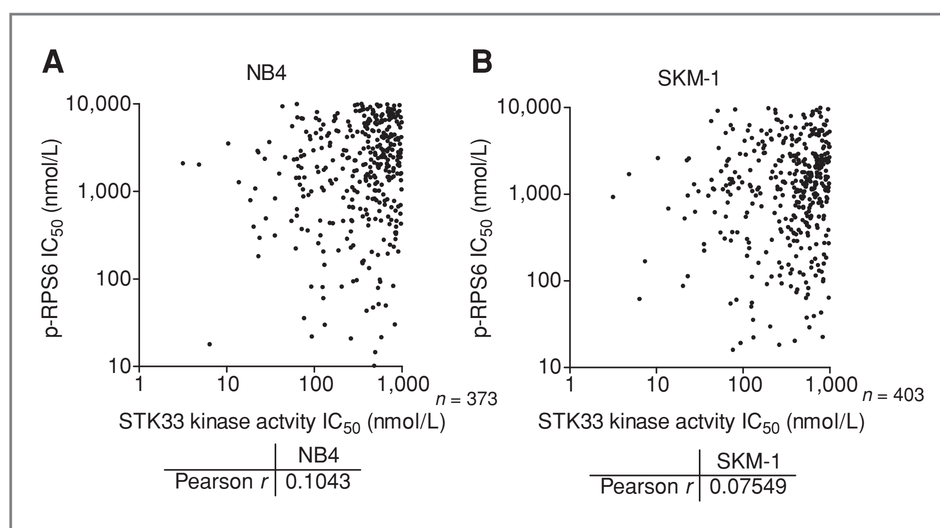

Figure 5. No correlation between inhibition of STK33 kinase activity and inhibition of RPS6 phosphorylation in mutant KRAS cell lines. A, the 510 most potent STK33 kinase inhibitors were tested for their ability to inhibit RPS6 phosphorylation in NB4 cells. The IC_{50} of each molecule on STK33 kinase activity is plotted on the *x*-axis and the IC_{50} of each molecule on RPS6 phosphorylation is plotted on the *y*-axis. The correlation between the 2 variables was calculated using the Pearson's correlation coefficient equation. Molecules that showed no evidence of inhibition of p-RPS6 phosphorylation (perhaps due to their lack of cell permeability) were eliminated from the analysis to avoid bias toward a less significant correlation. The statistics were calculated based on 373 molecules for NB4 and 403 molecules for SKM-1 cells. B, same as A, except that SKM-1 cells were used for the RPS6 phosphorylation assay.

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Small molecule STK33 kinase inhibitors do not selectively inhibit the viability of KRAS-dependent cell lines

The STK33 kinase inhibitors were used to determine whether STK33 inhibition has any effect on the phosphorylation of p70S6K or RPS6 in cells. Our preliminary experiments showed that the levels of p70S6K phosphorylation are very low in many cell lines (Fig. 1A and C; Supplementary Fig. S4) and are therefore not amenable for quantitative assays. We therefore decided to measure the levels of RPS6 phosphorylation (pRPS6), a well known p70S6K substrate which is inhibited when mutant KRAS is downregulated (Fig. 1A and C). The 510 most potent STK33 kinase inhibitors were tested for their ability to inhibit the phosphorylation of RPS6 in 3 cell lines, 2 with mutant KRAS (NB4 and SKM-1) and 1 wild-type for KRAS as a control (U-87 MG). Potent inhibition of RPS6 phosphorylation (<100 nmol/L) was observed in all 3 cell lines (Fig. 5A and B and Supplementary Table S1) showing that these compounds are cell permeable. However, our results showed no correlation between the effect of the small molecules tested on STK33 kinase activity in the biochemical assay and their effect on the phosphorylation of RPS6 in any of the cell lines tested (Fig. 5A and B; Supplementary Table S1).

Finally, the 145 most potent STK33 kinase inhibitors were tested in 4 AML cell lines, 2 mutant (NB4 and SKM-1) and 2 wild-type (OCI-AML3 and U937) for KRAS, for their effects on cell viability. First, our data showed no significant correlation between the potency of the compounds on the STK33 kinase activity and on cell viability (Fig. 6A–D; Supplementary Table S2). Second, the effects observed were independent of KRAS mutational status (Fig. 6A–E). Together, our data do not support a synthetic lethal relationship between KRAS dependence and STK33.

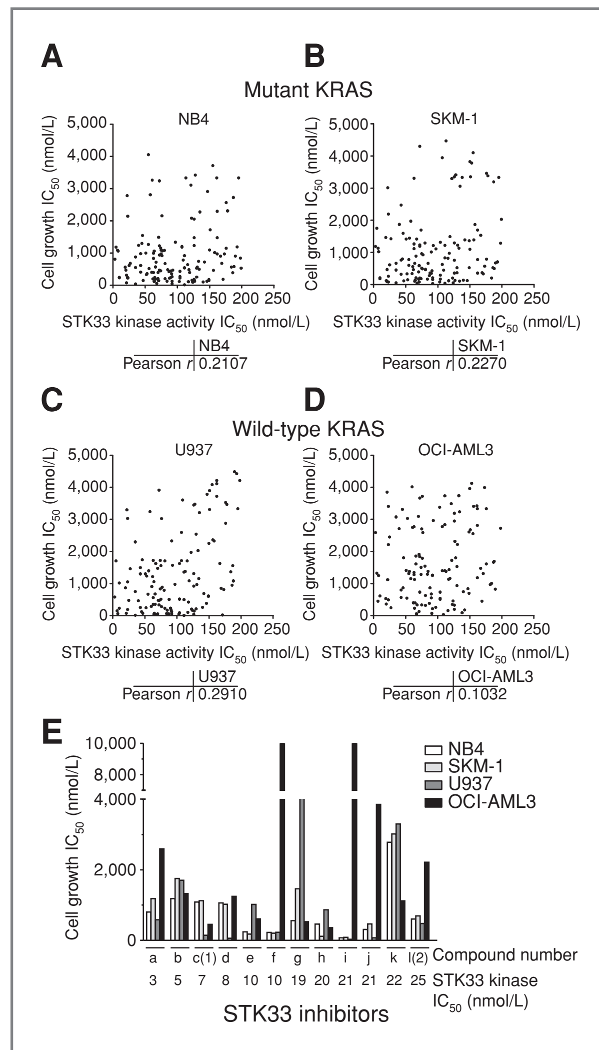

Figure 6. Small molecule STK33 kinase inhibitors do not selectively inhibit the growth of KRAS-dependent cell lines. A and B, the 145 most potent STK33 kinase inhibitors were tested for their ability to inhibit the viability of mutant KRAS AML cell lines (A, NB4 cells and B, SKM-1 cells). Each cell line was tested with the 145 molecules twice (NB4 and U937) or 3 times (SKM-1 and OCI-AML3) on different days with similar results. The IC_{50} of each molecule on STK33 kinase activity is plotted on the *x*-axis and the IC_{50} of each molecule on cell growth is plotted on the *y*-axis. The correlation between the 2 variables was calculated using the Pearson's correlation coefficient equation. C and D, same as A and B, except in wild-type KRAS cell lines (C, U937 cells and D, OCI-AML3 cells). E, the activity of 12 potent STK33 kinase inhibitors on the growth of 4 cell lines is illustrated. Data are from one representative experiment. The IC_{50} for each compound on STK33 kinase activity is indicated below the *x*-axis. Compound 1(c) and compound 2(l) are the same molecules as presented in Fig. 4D.

Discussion

KRAS mutations are an important driver of tumor growth. In the past 15 years, the inhibition of driver mutations, such as those found in several kinases including ABL, KIT, epidermal growth factor receptor, and B-Raf, has resulted in important clinical advances (6–10). However, targeting of other important driver genes such as KRAS has remained elusive. It has long been recognized that blocking the deleterious effects of KRAS mutations would likely be an effective approach to treat cancers that carry KRAS mutations. Because KRAS itself has been difficult to target, several groups have looked for alternative approaches to block KRAS function. Several potential targets have been described recently, mostly through screens for synthetic lethal interactions (3, 11, 12). We decided to focus on one of these novel targets, STK33, because its kinase activity was documented to be required for the survival of KRAS-dependent cell lines.

This work describes 3 different methods used to study the role of STK33 in cancer cell lines: RNAi, overexpression of wild-type or K145M STK33, and small molecule STK33 kinase inhibitors. In our hands, downregulation of STK33 expression by RNAi had little effect on the survival of multiple mutant KRAS cell lines. The lack of effect of STK33 knockdown on the survival in NOMO-1 and SKM-1 cell lines was consistent with the lack of effect on 2 putative STK33 downstream signaling proteins, p70S6K and RPS6, in these same cell lines. Our results are in contrast to those of Scholl and colleagues, who showed that knockdown of STK33 in these same cell lines decreased both p70S6K phosphorylation and cell viability (3). The reason for the discrepancy between the 2 studies is not

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likely due to a difference in cell lines. We have confirmed by DNA sequencing that NOMO-1 and SKM-1 cells harbor KRAS mutations (G13D and K117N, respectively). Moreover, KRAS knockdown in these cell lines inhibits p70S6K and RPS6 phosphorylation and cell survival. On the other hand, it is possible that differences in assay conditions could account for the different results.

It is interesting to note that STK33 was a hit in a short hairpin RNA (shRNA) screen to identify essential kinases in 293T cells, which are not KRAS dependent (13). On the other hand, STK33 was not identified in another synthetic lethal screen for KRAS dependency (12), or in our screens (Fig. 2). Knockdown of KRAS in our screens readily identified KRAS-dependent cell lines (Fig. 2), showing the robustness of the screens. False-negative results are not uncommon in si/shRNA screens and may be due to the inability to efficiently downregulate certain genes in high-throughput experiments. Our data confirmed the efficient knockdown of both STK33 and KRAS (Supplementary Fig. 2A and C), but also suggested off-target effects for 2 siRNAs, STK33_2Q and STK33_7Q, which inhibited cell viability (Supplementary Figs. S2 and S3). The off-target effects of STK33_7Q were not apparent in NOMO-1 and SKM-1 cells (Fig. 1A and C), but these findings illustrate how false-positive results can easily occur. False positives are not uncommon in si/shRNA screens because of the off-target effects of both siRNAs and shRNAs and the methods used to analyze the data (14). The algorithm used in our analysis is designed to avoid false positives associated with single siRNAs that give off-target effects. Our robust analysis method might also account for the discordance between our data and those previously published.

P70S6K and RPS6 phosphorylation was not affected in multiple cell lines despite efficient knockdown of STK33 and efficient overexpression of a kinase dead STK33. Moreover, across 510 small molecule STK33 kinase inhibitors, there was no correlation between their effect on STK33 kinase activity and their effect on the phosphorylation of RPS6 in any of the cell lines tested. This is not due to a failure of the assay because the most potent inhibitors of RPS6 phosphorylation across all 3 cell lines tested were potent PI3Kα/mTOR inhibitors (data not shown). PI3Kα/mTOR inhibitors are known to inhibit RPS6 phosphorylation (15). Together these data suggest that p70S6K and RPS6 are not substrates for STK33. During the course of our studies, we carried out several experiments to identify additional STK33 substrates without success. First, a ProtoArray of more than 9,000 full-length proteins was screened using recombinant wild-type versus K145M STK33 proteins, but no clear substrates were identified (data not shown). It is interesting that a p70S6K peptide that contains threonine 389, the same amino acid whose phosphorylation was shown to be modulated by STK33 downregulation by Scholl and colleagues (3), is a substrate for STK33 in our biochemical enzyme assay. However, the full-length p70S6K was not recognized by STK33 in the ProtoArray or modulated by STK33 knockdown in mutant KRAS cell lines in our studies. There are many examples where peptides are substrates for kinases *in vitro* even though the full-length protein is not a biological substrate. Second, lysates from wild-type or K145M STK33 overexpressing PANC-1 or 293T cells were used to probe 46 intracellular serine–threonine–tyrosine phosphosites. No STK33-dependent phosphorylation events were observed in either cell line (data not shown).

Finally, if STK33 kinase activity is important for the survival of mutant KRAS cell lines, small molecule STK33 kinase inhibitors would be expected to inhibit cell viability in a mutant KRAS-dependent manner. The 145 inhibitors that we tested inhibited cell viability with a range of potencies that showed no statistical correlation with STK33 kinase activity or KRAS dependency. The effects observed on cell viability are likely due to inhibition of kinases other than STK33.

In summary, our data show that STK33 kinase activity is not essential for the survival of KRAS-dependent AML cell lines. Further investigation will be necessary to define whether kinase-independent functions of STK33 are important in KRAS-dependent tumors. Moreover, identification of a direct substrate for STK33 will be critical to determine the exact relationship between the inhibition of STK33 kinase activity in cancer cells and a functional outcome.

Disclosure of Potential Conflicts of Interest

All authors are employees and shareholders of Amgen Inc.

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