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Synthetic Lethal Interaction between Oncogenic *KRAS* Dependency and *STK33* Suppression in Human Cancer Cells

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SUMMARY

An alternative to therapeutic targeting of oncogenes is to perform "synthetic lethality" screens for genes that are essential only in the context of specific cancer-causing mutations. We used high-throughput RNA interference (RNAi) to identify synthetic lethal interactions in cancer cells harboring mutant *KRAS*, the most commonly mutated human oncogene. We find that cells that are dependent on mutant *KRAS* exhibit sensitivity to suppression of the serine/threonine kinase STK33 irrespective of tissue origin, whereas *STK33* is not required by *KRAS*-independent cells. STK33 promotes cancer cell viability in a kinase activity-dependent manner by regulating the suppression of mitochondrial apoptosis mediated through S6K1-induced inactivation of the death agonist BAD selectively in mutant *KRAS*-dependent cells. These observations identify *STK33* as a target for treatment of mutant *KRAS*-driven cancers and demonstrate the potential of RNAi screens for discovering functional dependencies created by oncogenic mutations that may enable therapeutic intervention for cancers with "undruggable" genetic alterations.

INTRODUCTION

The identification of genes that are causally implicated in human cancer has resulted in novel, pathogenesis-oriented treatment strategies (Sawyers, 2004). Many known oncogenes, however, are challenging therapeutic targets. For example, efforts to develop drugs that inhibit oncogenic RAS proteins have been largely unsuccessful, despite the fact that *RAS* gene family members are mutated in approximately 30% of human tumors and that cancer cells are dependent on mutant *RAS* for their viability and proliferation (Karnoub and Weinberg, 2008; Roberts and Der, 2007).

As a result of an oncogenic mutation, cancer cells may also develop secondary dependencies on genes that are themselves not oncogenes. Perturbation of these genes may result in oncogene-specific "synthetic lethal" interactions that could provide new therapeutic opportunities (Hartwell et al., 1997; Kaelin, 2005). Synthetic lethality occurs when alteration of a gene results in cell death only in the presence of another nonlethal genetic alteration, such as a cancer-associated mutation. Mechanistically, synthetic lethal interactions can involve genes within the same pathway, genes within parallel pathways that cooperate with respect to an essential function, or genes within distant pathways that become functionally connected because of the response of the cell to a specific perturbation. Synthetic lethal interactions were first described in model organisms

(Bender and Pringle, 1991; Lucchesi, 1968), but more recent studies indicate that the concept of synthetic lethality can be extended to mammalian cells (Simons et al., 2001; Stockwell et al., 1999).

A strategy for identifying such genetic interdependencies in human cancer is to systematically determine the functional consequences of gene suppression in cancer cell lines using RNA interference (RNAi) technology (Bernards et al., 2006; Downward, 2004; Westbrook et al., 2005). For example, an RNAi screen for genes that are differentially required among cell lines representing molecular variants of diffuse large B cell lymphoma (DLBCL) identified *CARD11* as a regulator of constitutive NFKB signaling in the activated B cell-like DLBCL subtype (Ngo et al., 2006). Similarly, functional genetic screens have identified genes whose suppression sensitizes cancer cell lines (Turner et al., 2008; Whitehurst et al., 2007) or untransformed cells engineered to ectopically express a specific oncogene (Rottmann et al., 2005) to the effects of defined environmental conditions, such as the presence of a therapeutic agent.

The goal of this study was to identify synthetic lethal genetic interactions in the context of mutant *KRAS*, the most commonly mutated human oncogene. Based on high-throughput RNAi screens in human cancer cell lines that were categorized according to the presence or absence of a transforming *KRAS* mutation, we identified and functionally validated a serine/threonine protein kinase, STK33, that is selectively required for the survival and proliferation of mutant *KRAS*-dependent cancer cells across a wide range of tissue contexts. These findings demonstrate the potential of functional genetic approaches for identifying genotype-specific lethal genes in human cancer cells and support *STK33* as a target for treatment of the broad spectrum of human cancers associated with mutant *KRAS*.

RESULTS

Identification of a Synthetic Lethal Interaction between Mutant *KRAS* and Suppression of *STK33* with Large-Scale RNAi Screens

To identify genes that are essential for cancer cell viability and proliferation, we performed high-throughput loss-of-function RNAi screens in eight human cancer cell lines, representing five tumor types (Table 1), as well as normal human fibroblasts and immortalized human mammary epithelial cells (HMECs). We screened each cell line with a subset of the Broad Institute TRC shRNA Library that consists of 5024 short hairpin RNA (shRNA) constructs targeting 1011 human genes, including the majority of known and putative protein kinase genes and a selection of protein phosphatase genes and known cancer-related genes (Table S1 available online).

To identify genes that are required specifically in the context of mutant *KRAS*, we used a two-step approach (Figure 1A). We first compared the screening results obtained in *KRAS* mutant NOMO-1 acute myeloid leukemia (AML) cells with those obtained in THP-1 (a *KRAS* wild-type [WT] AML cell line), fibroblasts, and HMECs to identify shRNAs that selectively impaired viability and proliferation in NOMO-1 cells. We used supervised analysis to rank these shRNAs and identified a list of genes for which multiple shRNAs with efficient target gene suppression (as assessed by high-throughput quantitative RT-PCR) affected NOMO-1 but not THP-1 or untransformed cells. To determine which of these genes were selectively required in a broader spectrum of *KRAS* mutant cancer cell lines, we then evaluated shRNAs targeting the top-ranking candidate genes in the remaining six cell lines (*KRAS* mutant, n = 3; *KRAS* WT, n = 3) that were included in the primary RNAi screens. shRNAs targeting *STK33* ($p = 0.003$), which encodes a putative member of the calcium/calmodulin-dependent protein kinase subfamily of serine/threonine protein kinases (Mujica et al., 2001), and shRNAs targeting *KRAS* itself ($p = 0.004$) yielded the strongest evidence of selective impairment in viability and proliferation in *KRAS* mutant cell lines (Figures 1B and 1C). Consistent with the results of high-throughput quantitative RT-PCR, the toxicity of shRNAs targeting *STK33* and *KRAS* was associated with target gene suppression, thus confirming the specificity of the screening results (Figure S1). Taken together, these observations indicated a synthetic lethal interaction between the presence of a functionally relevant *KRAS* mutation and suppression of *STK33*.

Identification of Transforming *KRAS* Mutations in AML Cell Lines Based on Sensitivity to *STK33* Suppression

To verify the selective requirement for *STK33* in mutant *KRAS*-dependent cells, we analyzed the effects of suppressing *STK33* in 7 AML cell lines (Figure 2A). As predicted by the RNAi screens, shRNAs targeting *STK33* and *KRAS* had a strong antiproliferative effect on NOMO-1 but not THP-1 cells. Unexpectedly, knockdown of *KRAS* and *STK33* also inhibited the viability and proliferation of two cell lines that have not been reported to harbor mutant *KRAS*, NB4 and SKM-1. To explore this disparity, we performed DNA sequence analysis of the *KRAS* coding region and found that both NB4 and SKM-1 cells harbor missense mutations in *KRAS* (c.53C→A, p.A18D; c.351A→C, p.K117N) (Figure 2B). In contrast, no *KRAS* mutations were detected in the *STK33*- and *KRAS*-independent cell lines THP-1, Mono-Mac-6, OCI-AML3, and U937.

Several studies indicate that mutations in codons 18 and 117 of *RAS* family members may play a role in tumorigenesis. *KRAS* codon 18 mutations have been detected in patients with lung adenocarcinoma, colorectal adenoma, and adrenocortical tumors (Lin et al., 1998; Suzuki et al., 1990; Wang et al., 2006) and are associated with decreased GTPase activity (Lin et al., 2000); mutations in codon 18 of *NRAS* and *HRAS* have been observed in patients with malignant melanoma and metastatic pituitary carcinoma, respectively (Demunter et al., 2001a, 2001b; Pei et al., 1994). The KRAS K117N allele occurs in human colorectal cancer (Wood et al., 2007), and activating mutations involving *HRAS* codon 117 have been reported in Costello syndrome, a developmental disorder associated with various cancers (Schubbert et al., 2007), and a human multiple myeloma cell line (Crowder et al., 2003).

To directly assess the transforming potential of the KRAS A18D and K117N alleles, we investigated their functional consequences in NIH/3T3 murine embryonic fibroblasts and BaF3 murine pro-B cells. Expression of KRAS A18D or K117N in NIH/3T3 cells resulted in anchorage-independent growth in soft agar (Figure 2C) and loss of contact inhibition in focus

Table 1. Selective Sensitivity of Mutant *KRAS*-Dependent Cancer Cell Lines to Suppression of *STK33*

Cancer Type	Cell Line	*KRAS* Status[b]	*KRAS* Dependency	*STK33* Dependency
AML	NOMO-1[a]	Mutant (G13D)	Yes[c,d]	Yes[c,d]
	NB4	Mutant (A18D)	Yes[d]	Yes[d]
	SKM-1	Mutant (K117N)	Yes[d]	Yes[d]
	Mono-Mac-6	Wild-type	No[d]	No[d]
	OCI-AML3	Wild-type	No[d]	No[d]
	THP-1[a]	Wild-type	No[c,d]	No[c,d]
	U937	Wild-type	No[d]	No[d]
Multiple myeloma	RPMI-8226	Mutant (G12A)	Yes[d]	Yes[d]
	MM.1S	Wild-type	No[d]	No[d]
Breast cancer	MDA-MB-231[a]	Mutant (G13D)	Yes[c,e,f]	Yes[c,e,f]
	MDA-MB-453[a]	Wild-type	No[c]	No[c]
	BT20	Wild-type	No[f]	No[f]
Colon cancer	DLD-1[a]	Mutant (G13D)	Yes[c,e,f]	Yes[c,e,f]
	HCT-116[a]	Mutant (G13D)	No[c]/Yes[e]	Yes[c,e]
	SW-480	Mutant (G12V)	Yes[e,f]	Yes[e,f]
	HCT-15	Mutant (G13D)	No[e,f]	No[e,f]
	COLO-320 HSR	Wild-type	No[e,f]	No[e,f]
Pancreatic cancer	PANC-1	Mutant (G12D)	Yes[e,f]	Yes[e,f]
	Bx-PC3	Wild-type	No[f]	No[f]
Lung cancer	A549	Mutant (G12S)	Yes[e,f]	Yes[e,f]
Glioblastoma	U-87-MG[a]	Wild-type	No[c]	No[c]
Prostate cancer	PC-3[a]	Wild-type	No[c]	No[c]
T-ALL	CCRF-CEM	Mutant (G12D)	No[d]	No[d]
	P12-Ichikawa	Wild-type	No[d]	No[d]
	Jurkat	Wild-type	No[d]	No[d]

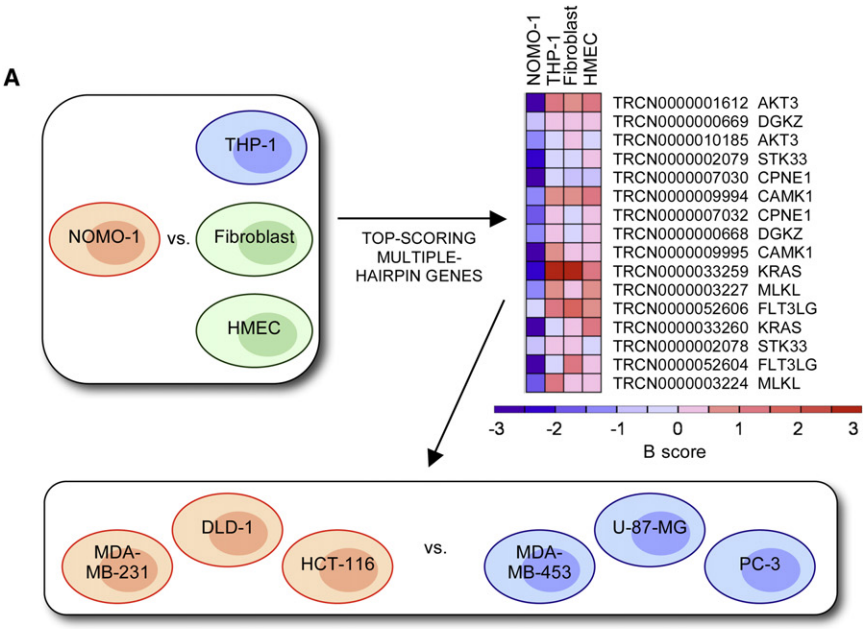
[a] Cell lines screened by high-throughput RNAi.
[b] Sequence numbering is according to NCBI Reference Sequences NP_004976.2 and NP_203524.1.
[c] As assessed by high-throughput RNAi.
[d] As assessed in cell viability assays.
[e] As assessed by colony formation in soft agar.
[f] As assessed by tumor formation in immunocompromised mice.

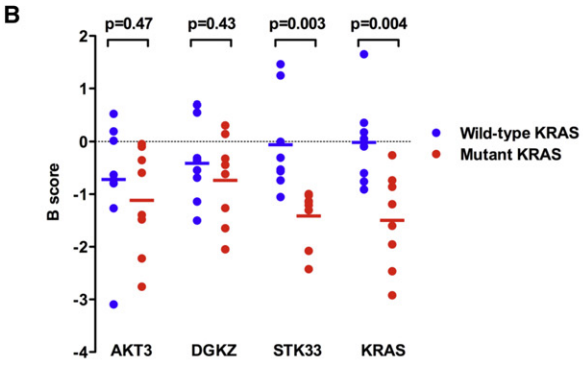
formation assays (Figures 2D and S2A). Furthermore, injection of NIH/3T3 cells expressing either allele into immunocompromised mice led to tumor formation in vivo (Figure S2B). In addition, expression of KRAS A18D and K117N conferred cytokine independence to BaF3 cells (Figure 2E). Considered together, these results indicate that KRAS A18D and K117N are gain-of-function mutations with transforming activity and provided further support for a synthetic lethal relationship between *STK33* inactivation and the presence of a functionally relevant *KRAS* mutation.

In agreement with our observations in AML cell lines, *STK33* was also required by a mutant *KRAS*-dependent multiple myeloma cell line, RPMI-8226, whereas *STK33* knockdown had only a marginal effect in a *KRAS* WT multiple myeloma cell line, MM.1S (Figure 2F). Finally, *STK33* suppression had no effect in *KRAS* WT T cell acute lymphoblastic leukemia (T-ALL) cell lines, as well as in a T-ALL cell line that harbors a KRAS G12D substitution but is insensitive to *KRAS* knockdown (Figure 2G), again suggesting that *STK33* is preferentially required by cells that are dependent on mutant *KRAS*.

Synthetic Lethal Interaction between Mutant *KRAS* Dependency and Suppression of *STK33* in Epithelial Cancer Cell Lines

To determine whether our finding of a correlation between mutant *KRAS* dependency and sensitivity to *STK33* knockdown could be extrapolated to cancers that occur outside the hematopoietic system, we investigated the effects of *STK33* suppression in different epithelial cancer cell lines. shRNA knockdown of *STK33* impaired colony formation in semisolid medium by mutant *KRAS*-dependent HCT-116 and SW-480 colon cancer cells, MDA-MB-231 breast cancer cells, PANC-1 pancreatic cancer cells, and A549 lung cancer cells with similar efficiency as did knockdown of *KRAS*. In contrast, there was no effect of *STK33* knockdown on anchorage-independent growth of *KRAS* WT COLO-320 HSR colon cancer cells (Figure 3A). To confirm these observations in vivo, we transduced seven epithelial cancer cell lines with shRNA constructs targeting *STK33*. Suppression of *STK33* decreased the ability of SW-480, PANC-1, MDA-MB-231, and A549 cells to form tumors in immunocompromised mice. In contrast, *STK33* knockdown had no





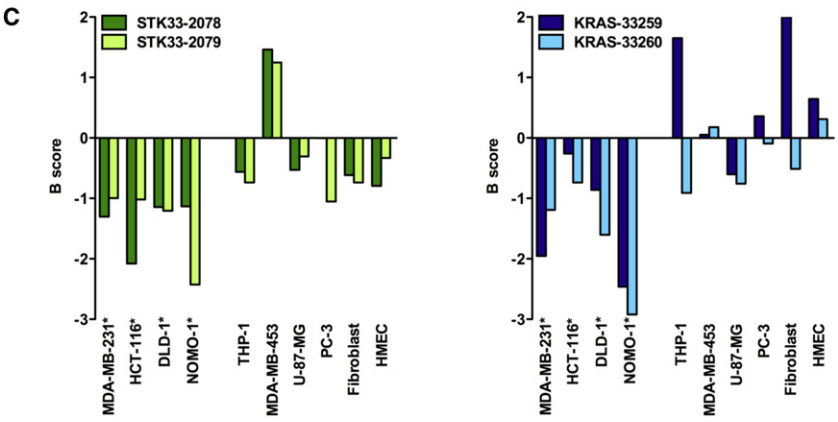


Figure 1. RNAi Screens for Genes Required by *KRAS* Mutant Cancer Cells

(A) Experimental strategy. Data obtained in *KRAS* mutant NOMO-1 cells were compared with data obtained in *KRAS* WT THP-1 cells, fibroblasts, and HMECs. Genes selectively required by NOMO-1 cells were identified on the basis of supervised analysis of shRNA performance, and shRNAs targeting the top-ranking candidate genes were evaluated in additional cancer cell lines included in the primary RNAi screens. Cell lines with or without mutant *KRAS* are indicated in orange and blue, respectively; untransformed cell types are indicated in green.

(B) Preferential sensitivity of *KRAS* mutant cell lines to *STK33* suppression. The effects of two shRNAs targeting each of the top-ranking candidate genes in eight cancer cell lines are shown. Bars indicate mean values. Statistical significance was assessed via the unpaired t test.

(C) Effects of *STK33* and *KRAS* suppression in eight cancer cell lines and two untransformed cell types. *KRAS* mutant cell lines are marked with an asterisk.

effect in COLO-320 HSR cells, as well as *KRAS* WT BxPC-3 pancreatic cancer cells and BT20 breast cancer cells (Figures 3B, S3, and S4).

Another demonstration of the functional relationship between mutant *KRAS* and *STK33* dependency came from results obtained in HCT-15, a sister cell line of DLD-1 colon cancer cells (Figure 3C). In agreement with previous observations (Shirasawa et al., 1993), DLD-1 cells were dependent on *KRAS*, and in consonance with our screening data, *KRAS* dependency was associated with sensitivity to *STK33* knockdown. In contrast, HCT-15 cells did not require *KRAS*, even though they are derived from the same patient as DLD-1 cells and carry the identical *KRAS* mutation (Chen et al., 1995), consistent with a recent report showing lower constitutive KRAS activity in HCT-15 cells as compared to DLD-1 (Smakman et al., 2006). Strikingly, the lack of *KRAS* dependency of HCT-15 cells was paralleled by resistance to *STK33* suppression.

Overall, we evaluated the functional consequences of *STK33* knockdown in 25 cell lines representing nine different tumor types (Table 1). These experiments demonstrated that *STK33* is preferentially required by cells that rely on mutant *KRAS* for their survival and proliferation, but not cells harboring WT *KRAS* or *KRAS*-independent cells, indicating that oncogenic mutations may create genotype-specific functional dependencies across different tumor types. These findings also suggest the possibility that targeting of *STK33* may provide a substantive therapeutic window in a broad spectrum of human cancers associated with mutant *KRAS*.

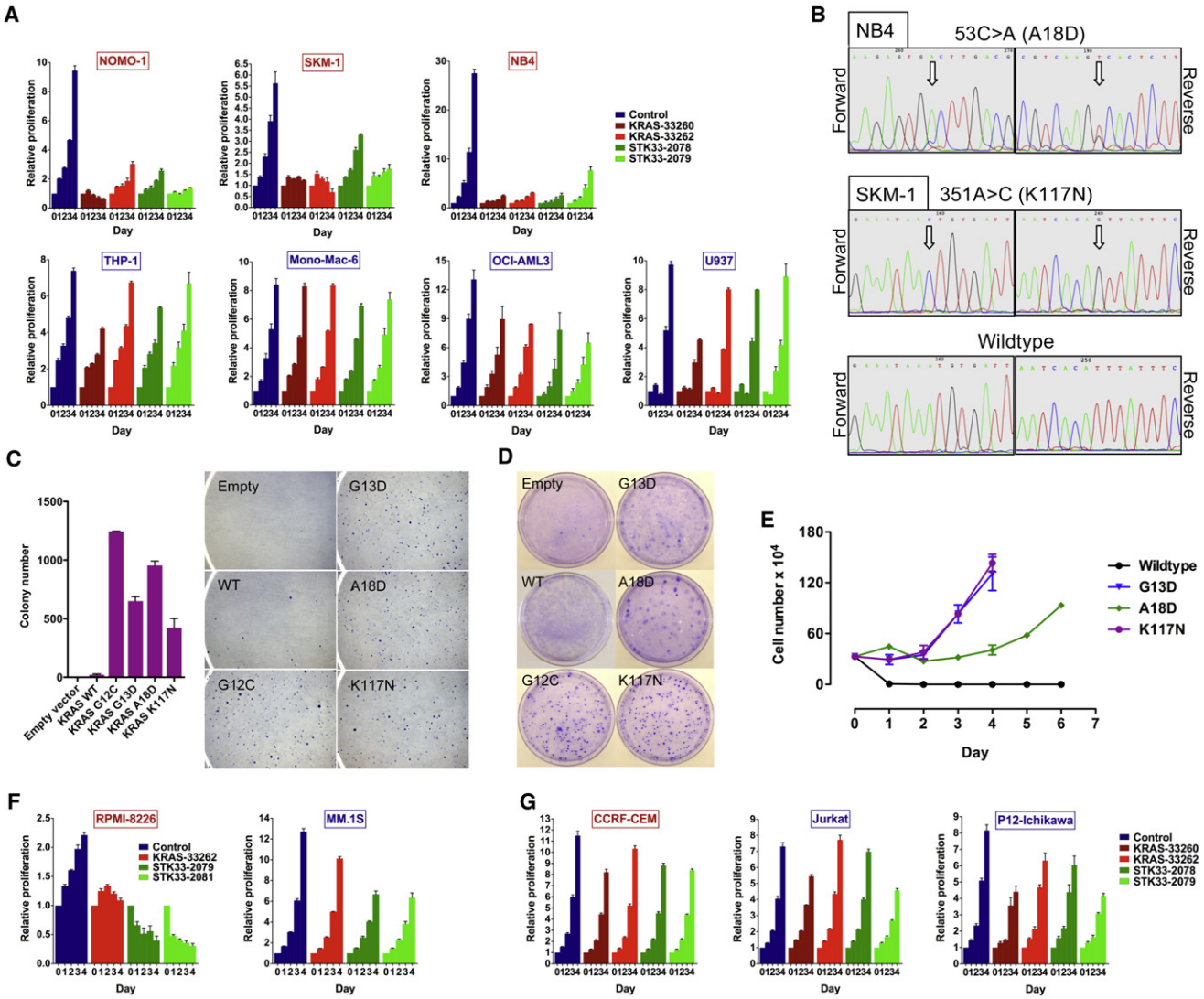


Figure 2. Selective Requirement for *STK33* in Mutant *KRAS*-Dependent Hematopoietic Cancer Cells

(A) Effects of *KRAS* and *STK33* knockdown on viability and proliferation of AML cell lines.

(B) Detection of *KRAS* mutations in NB4 and SKM-1 cells. Portions of *KRAS* coding exons 1 and 3 are shown. Nucleotide changes (arrows) and resulting amino acid substitutions (in parentheses) are given above the chromatograms; sequence numbering is according to NCBI Reference Sequences NM_033360 and NP_203524. Chromatograms showing part of the WT sequence of *KRAS* coding exon 3 are provided for comparison.

(C) Anchorage-independent growth of NIH/3T3 cells expressing KRAS A18D and K117N. Colony numbers and photographs of crystal violet-stained colonies are shown. Duplicate experiments, mean ±SEM.

(D) Loss of contact inhibition of NIH/3T3 cells expressing KRAS A18D and K117N.

(E) IL-3-independent growth of BaF3 cells expressing KRAS A18D and K117N.

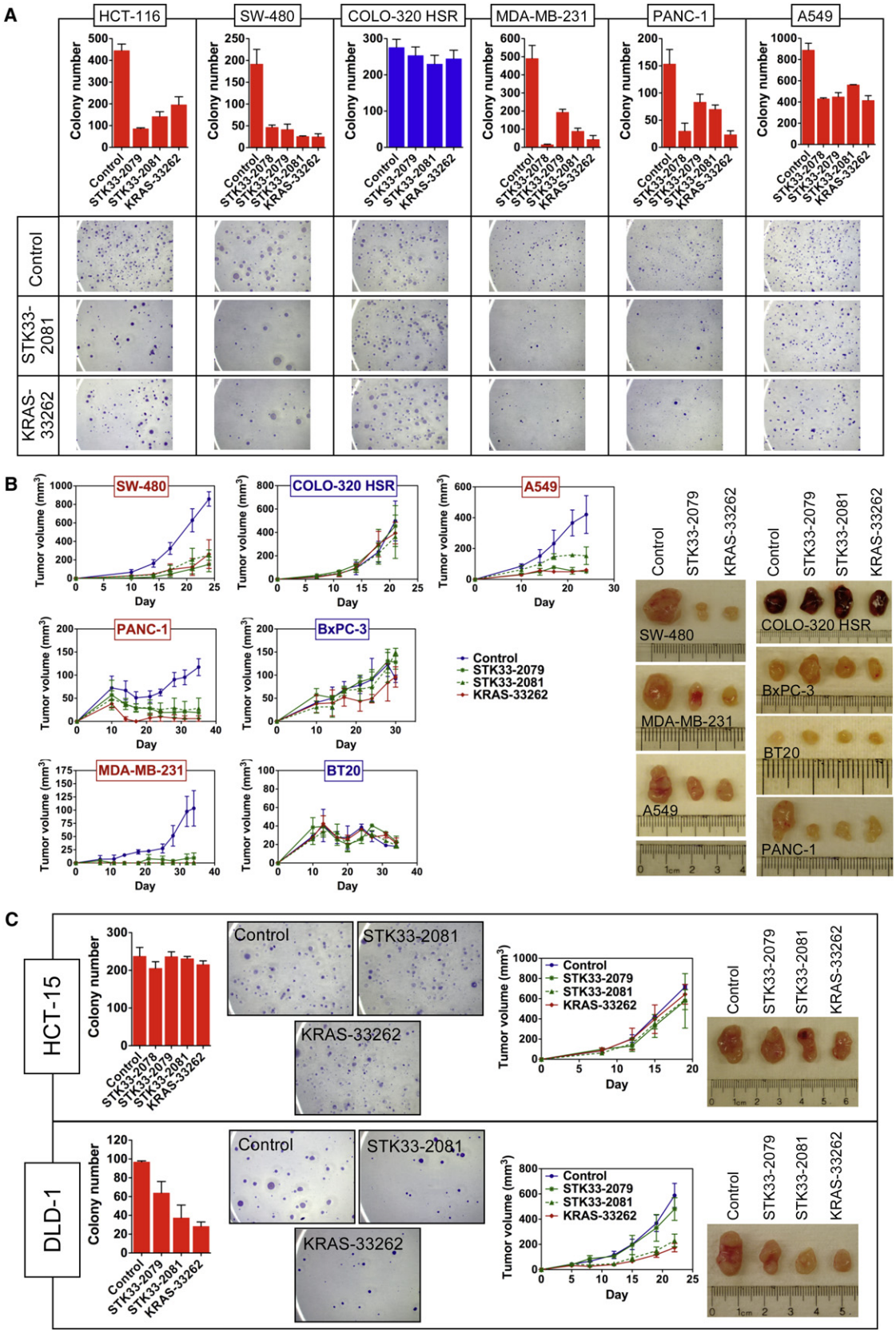
(F) Effects of *KRAS* and *STK33* knockdown on viability and proliferation of multiple myeloma cell lines.

(G) Effects of *KRAS* and *STK33* knockdown on viability and proliferation of T-ALL cell lines. Error bars in (A) and (E)–(G) represent the mean ±SEM of triplicate experiments. *KRAS* mutant cell lines in (A) and (F) – (G) are indicated in red.

Analysis of the molecular genetic profiles of the cell lines used in this study showed that sensitivity to *STK33* suppression is not conferred by mutant *NRAS*, alterations of genes involved in the regulation of RAS activity (e.g., *FLT3* and *PTPN11*), or mutational activation of RAS effector pathways such as the RAF-MEK-ERK and PI3K-AKT signaling cascades (Table S2). This supports the conclusion that *STK33* dependency represents a genotype-specific vulnerability of *KRAS* mutant cancer cells rather than a general functional characteristic of cells with elevated RAS activity.

Causal Relationship between Mutant *KRAS* Dependency and Requirement for STK33 Activity

Our observations suggested a causal relationship between the presence of a transforming *KRAS* mutation and the requirement for *STK33*. To test this hypothesis, we transduced 2 *KRAS*- and


A
HCT-116
SW-480
COLO-320 HSR
MDA-MB-231
PANC-1
A549
Colony number
Control
STK33-2079
STK33-2081
KRAS-33262
Control
STK33-2081
KRAS-33262
B
SW-480
COLO-320 HSR
A549
PANC-1
BxPC-3
MDA-MB-231
BT20
Tumor volume (mm3)
Day
Control
STK33-2079
STK33-2081
KRAS-33262
SW-480
COLO-320 HSR
BxPC-3
MDA-MB-231
BT20
A549
PANC-1
C
HCT-15
DLD-1
Colony number
Control
STK33-2081
KRAS-33262
Tumor volume (mm3)
Day
Control
STK33-2079
STK33-2081
KRAS-33262

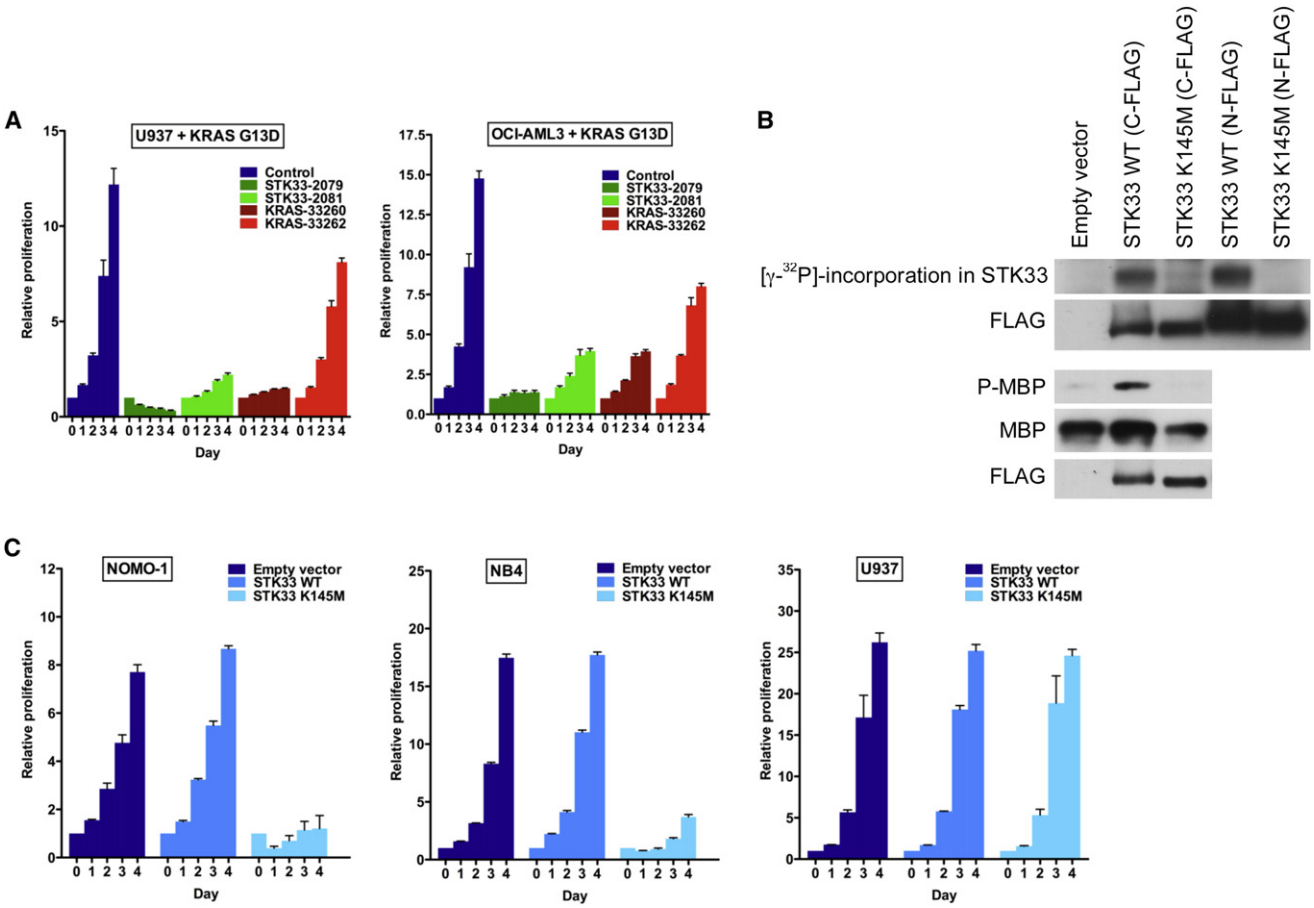


Figure 4. Induction of *STK33* Dependency by Mutant *KRAS* and Requirement for STK33 Activity in Mutant *KRAS*-Dependent AML Cells

(A) Acquired sensitivity to *STK33* and *KRAS* knockdown after exogenous expression of mutant *KRAS*. Triplicate experiments, mean ±SEM.

(B) Effect of the STK33 active site mutation K145M on STK33 kinase activity.

(C) Effect of exogenous expression of N-terminally FLAG-tagged STK33 K145M on cell viability and proliferation. Triplicate experiments, mean ±SEM.

STK33-independent AML cell lines, U937 and OCI-AML3, with a lentiviral vector encoding the KRAS G13D allele. Expression of KRAS G13D rendered these cell lines *KRAS* dependent, as evidenced by reduced cell viability and proliferation after *KRAS* knockdown. Of note, the newly acquired *KRAS* dependency was paralleled by sensitivity to *STK33* suppression (Figures 4A and S5). These findings support a role for mutant *KRAS* in establishing *STK33* dependency in human cancer cells.

To further elucidate the effects of *STK33* on cancer cell viability and proliferation, we evaluated the functional impact of kinase-deficient STK33 in human AML cells lines. Exogenous expression of an STK33 mutant in which a conserved lysine residue in the ATP-binding loop is changed to methionine (Varjosalo et al., 2008) inhibited viability and proliferation in mutant *KRAS*-dependent NOMO-1 and NB4 cells, whereas *KRAS* WT U937 cells were unaffected (Figures 4B and 4C). These observations are consistent with the hypothesis that the catalytic activity of STK33 is requisite for the survival and proliferation of mutant *KRAS*-dependent cells and indicate that *STK33* may be a promising target for therapy of cancers with oncogenic *KRAS* alleles.

Absence of Genomic Alterations of *STK33* in Human Cancer

We next explored whether the increased sensitivity of mutant *KRAS*-dependent cells to *STK33* suppression could be attributed to deregulated expression or structural alterations of the

Figure 3. Selective Requirement for *STK33* in Mutant *KRAS*-Dependent Epithelial Cancer Cells

(A) Effects of *STK33* and *KRAS* knockdown on soft agar colony formation of epithelial cancer cell lines. Colony numbers (red bars, *KRAS* mutant cell lines) and photographs of crystal violet-stained colonies are shown. Duplicate experiments, mean ±SEM.

(B) Effects of *STK33* and *KRAS* knockdown on tumor formation of epithelial cancer cell lines in *nude* mutant or NOD/SCID mice. Tumor volumes and photographs of tumors are shown. Two to six independent experiments, mean ±SEM *KRAS* mutant cell lines are indicated in red.

(C) Effects of *STK33* and *KRAS* knockdown on soft agar colony formation (colony numbers and photographs of crystal violet-stained colonies are shown; duplicate experiments, mean ±SEM) and tumor formation in *nude* mutant mice (tumor volumes and photographs of tumors are shown; two to four independent experiments, mean ±SEM) of *KRAS* mutant HCT-15 and DLD-1 colon cancer cells.

STK33 gene, located on chromosome band 11p15.3, that would be beneficial in the context of a transforming *KRAS* mutation.

We first measured *STK33* mRNA levels in 20 human cancer cell lines (*KRAS* mutant, n = 10; *KRAS* WT, n = 10) and identified no significant difference in *STK33* expression between *KRAS* mutant and *KRAS* WT cell lines (Figures S6A and S6C). In addition, we analyzed RNA from various normal human tissues (Figure S6B) and observed that *STK33* was not overexpressed in cancer cells (Figure S6C). We next performed DNA sequence analysis of all *STK33* exons in six cancer cell lines that were dependent on *STK33* and mutant *KRAS* (NOMO-1, NB4, SKM-1, MDA-MB-231, PANC-1, A549) and identified no mutations that were predicted to change the amino acid sequence of the STK33 protein (data not shown). Lastly, a recent analysis of DNA copy number alterations in 763 human cancer cell lines (http://www.sanger.ac.uk/cgi-bin/genetics/CGP/cghviewer/CghHome.cgi), which included 17 of the 25 cell lines that were used in this study (*KRAS* mutant, n = 9; *KRAS* WT, n = 8), identified no high-level amplifications of the *STK33* locus.

To evaluate a potential correlation between mutant *KRAS* and genomic alteration of *STK33* in primary patient samples, we analyzed bone marrow or peripheral blood from patients with core-binding factor AML, a subtype of AML that is recurrently associated with mutant *KRAS* (Bowen et al., 2005). DNA sequence analysis identified activating *KRAS* mutations in six (9%) of 67 cases; however, gene expression profiling with DNA microarrays showed no significant difference in *STK33* expression between cases with or without mutant *KRAS* (Figure S7). In accordance with these results and consistent with our observations in cancer cell lines, a meta-analysis of published transcriptome data from patients with colon cancer (Koinuma et al., 2006), pancreatic cancer (Jimeno et al., 2008), and non-small cell lung cancer (Bild et al., 2006) with Oncomine (http://www.oncomine.org) revealed no significant differences in *STK33* expression between cases with or without mutant *KRAS*. Finally, searches of published DNA sequence data sets (Greenman et al., 2007; Jones et al., 2008; Parsons et al., 2008; Wood et al., 2007) indicated that *STK33* is not recurrently mutated in primary human cancer samples.

Considered together, these observations suggest that the adaptive changes resulting in *STK33* dependency of *KRAS* mutant cells involve the establishment of a neomorphic functional circuitry, rather than transcriptional or structural alterations of the *STK33* gene itself, and illustrate the potential of RNAi screens for the detection of synthetic lethal interactions in cancer cells that cannot be identified with other genomic technologies.

These findings also indicate that *STK33* does not function as an oncogene, which is typically activated by structural alterations or overexpressed to an extent that directly promotes tumorigenesis. In support of this hypothesis, exogenous expression of *STK33* did not transform NIH/3T3 or BaF3 cells (Figures S8A, S8B, and S8C), and the effects of *KRAS* suppression on viability and proliferation of mutant *KRAS*-dependent human cancer cell lines could not be reversed by overexpression of *STK33* (Figure S8D). This indicates that mutant KRAS and STK33 are not functionally redundant signaling elements that can substitute for each other in maintaining the transformed phenotype, reinforcing the conclusion that *KRAS* mutant cancer cells are codependent on the continued expression of both *KRAS* itself and *STK33*.

Modulation of S6K1 Activity by STK33 in Mutant *KRAS*-Dependent Cancer Cells

Since the biochemical properties and biological function of STK33 are unknown, we had no preexisting insight into the potential mechanism through which *STK33* might function in mutant *KRAS*-dependent cells. We therefore evaluated the effects of *STK33* suppression on signaling pathways that are frequently deregulated in cancer (Figure 5A). Specifically, we used activation state-specific antibodies to analyze components of the PI3K-AKT, MAPK, and mTORC1 pathways in AML cell lines transduced with shRNA constructs targeting *STK33*. These experiments showed that *STK33* downregulation had no effect on the phosphorylation status of PDK1, AKT, MEK, ERK, RSK, or mTOR. In contrast, *STK33* suppression in mutant *KRAS*-dependent cells—but not cells that lack mutant *KRAS*—decreased the phosphorylation of the S6K1 serine/threonine protein kinase (Figures 5B and S9).

To verify that *STK33* suppression also reduced the activity of S6K1, we determined the phosphorylation status of known S6K1 substrates. *STK33* knockdown decreased the phosphorylation of RPS6, a downstream effector of S6K1 required for ribosome biogenesis and protein synthesis that is frequently used as a surrogate marker of S6K1 activity (Ruvinsky and Meyuhas, 2006), selectively in mutant *KRAS*-dependent cells (Figure 5B). Decreased S6K1 and RPS6 phosphorylation was also observed in response to exogenous expression of kinase-deficient STK33, indicating that the catalytic activity of STK33 is essential for maintaining S6K1 activity in mutant *KRAS*-dependent cells (Figure 5C).

Suppression of Mitochondrial Apoptosis via STK33-Mediated Inactivation of BAD in Mutant *KRAS*-Dependent Cells

S6K1 is also known to phosphorylate the proapoptotic BH3-only protein BAD at serine 136, leading to its inactivation and suppression of mitochondrial apoptosis (Harada et al., 2001; Zha et al., 1996). Consistent with the hypothesis that STK33 regulates the survival function of S6K1 in mutant *KRAS*-dependent cells, *STK33* suppression abrogated BAD S136 phosphorylation in NOMO-1 and SKM-1 but not U937 and OCI-AML3 cells (Figure 6A). This was accompanied by selective induction of apoptosis in NOMO-1 and SKM-1, as assessed by quantification of annexin V-positive cells (Figure 6B), trypan blue staining (Figures 6C–6E), and detection of caspase 3 and poly(ADP-ribose) polymerase (PARP) cleavage (Figure 6A), an effect that could be partially overcome by pretreatment with the caspase inhibitor Z-VAD-FMK (Figure 6C).

Several findings demonstrated that apoptosis induced by *STK33* suppression was mediated via the mitochondrial pathway. First, *STK33* knockdown resulted in an increase in caspase 9 cleavage (Figure 6A). Second, exogenous *BCL2* expression protected mutant *KRAS*-dependent cells from the proapoptotic effects of *STK33* suppression (Figure 6D). Third, gene expression profiling in mutant *KRAS*-dependent AML cell lines expressing shRNAs targeting *STK33* revealed a significant

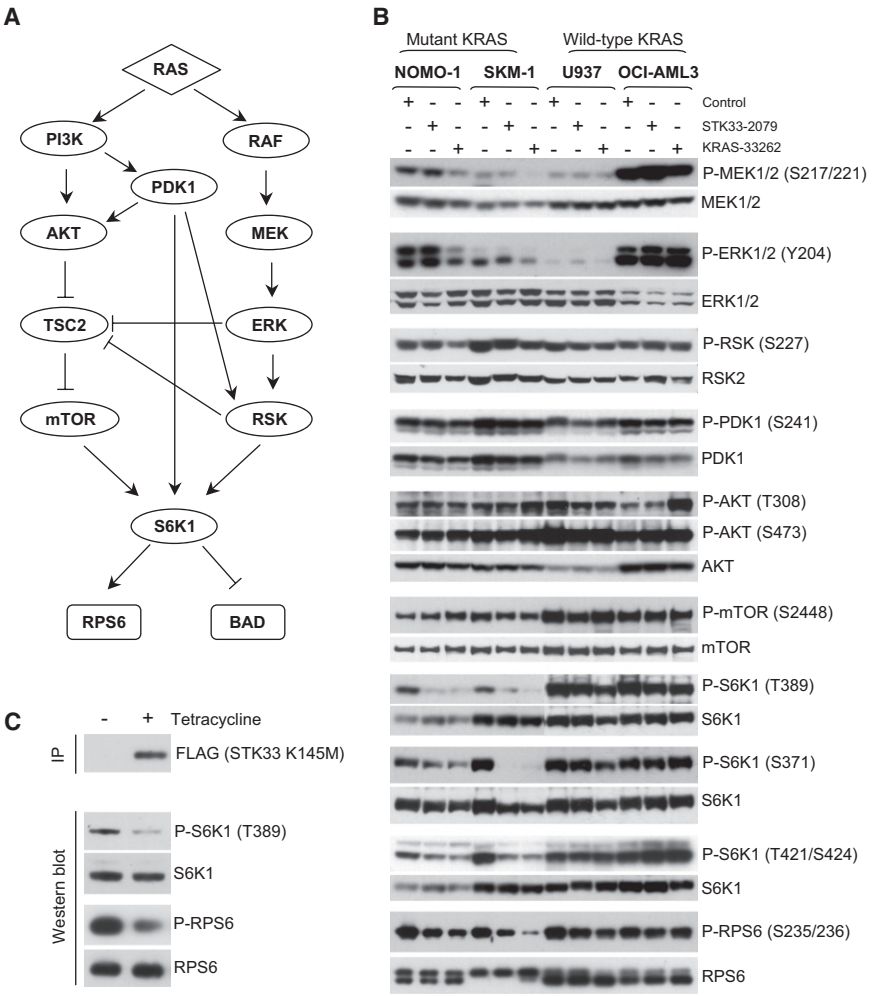


Figure 5. Selective Requirement for STK33 for S6K1 Activity in Mutant *KRAS*-Dependent Cancer Cells

(A) Schematic representation of the signaling pathways studied.

(B) Phosphorylation state of components of the MAPK, PI3K-AKT, and mTORC1 pathways in AML cell lines transduced with shRNA constructs targeting *STK33* or *KRAS*.

(C) Effect of exogenous expression of N-terminally FLAG-tagged STK33 K145M on S6K1 activity. Mutant *KRAS*-dependent PANC-1 pancreatic cancer cells were transduced with lentiviral vectors encoding the STK33 K145M allele and the tetracycline repressor, respectively. STK33 K145M expression was induced by addition of tetracycline, and S6K1 and RPS6 phosphorylation were determined by western blotting. Expression of STK33 K145M protein was confirmed by immunoprecipitation (IP) and western blotting.

enrichment of components of the mitochondrial apoptotic pathway among the genes that were differentially regulated in response to *STK33* suppression (Figure S10).

To confirm the role of BAD in cell killing induced by *STK33* suppression, we transduced mutant *KRAS*-dependent AML cell lines with shRNA constructs targeting *BAD*. Knockdown of *BAD* rescued cell viability after *STK33* suppression (Figure 6E), whereas the cytotoxicity of *STK33* suppression was not reversed by downregulation of another proapoptotic BH3-only protein, BID, illustrating the specificity of the functional relationship between STK33 and S6K1-mediated survival signaling via inactivation of BAD (Figure 6E). Finally, western blot analysis of other BH3-only proteins as well as pro- and antiapoptotic BCL2 homologs identified no abnormalities (Figure S11), reinforcing that *STK33* suppression affected specifically the S6K1-BAD signaling axis. Taken together, these observations indicate that STK33 promotes cancer cell viability in a genotype-specific manner by regulating the activity of S6K1 selectively in mutant *KRAS*-dependent cells. Furthermore, they show that the mechanism of cell death after *STK33* suppression involves engagement of the mitochondrial apoptotic pathway through induction of BAD.

Since S6K1 is a downstream effector of mTORC1, our findings suggested that STK33 might act within the PI3K-AKT cascade or the MAPK signaling pathway, which increase mTORC1 activity, or alternatively function as an mTORC1 substrate. Several observations, however, argued against these possibilities. First, *STK33* suppression had no effect on the phosphorylation status of components of the PI3K-AKT cascade, intermediates of the MAPK pathway, or mTOR, regardless of *KRAS* mutation status (Figure 5B). Second, the effects of *STK33* silencing on cell viability and S6K1 phosphorylation could not be reversed by knockdown of the TSC2 tumor suppressor, a negative regulator of mTORC1 (Figure S12). Third, we found no direct interaction between STK33 and mTOR (Figure S13). Fourth, *STK33* knockdown also reduced the activity of an mTORC1-independent S6K1 mutant (Schalm et al., 2005) (Figure S14). Finally, a recent study showed that the survival function of S6K1 mediated by BAD S136 phosphorylation cannot be inhibited by treatment with the mTORC1 inhibitor rapamycin (Djouder et al., 2007). On the basis of these observations, we favor a model whereby STK33 modulates S6K1 activity in mutant *KRAS*-dependent cells through a mechanism that does not involve mTORC1.

DISCUSSION

In this study, we used a systematic functional genetic approach to search for synthetic lethal interactions in cancer cells harboring oncogenic *KRAS* mutations, which occur in approximately 30% of human tumors and have thus far not proven to be amenable to therapeutic targeting. Our results show that cells that are dependent on mutant *KRAS* exhibit selective sensitivity to suppression of *STK33*, a gene that has not been linked to

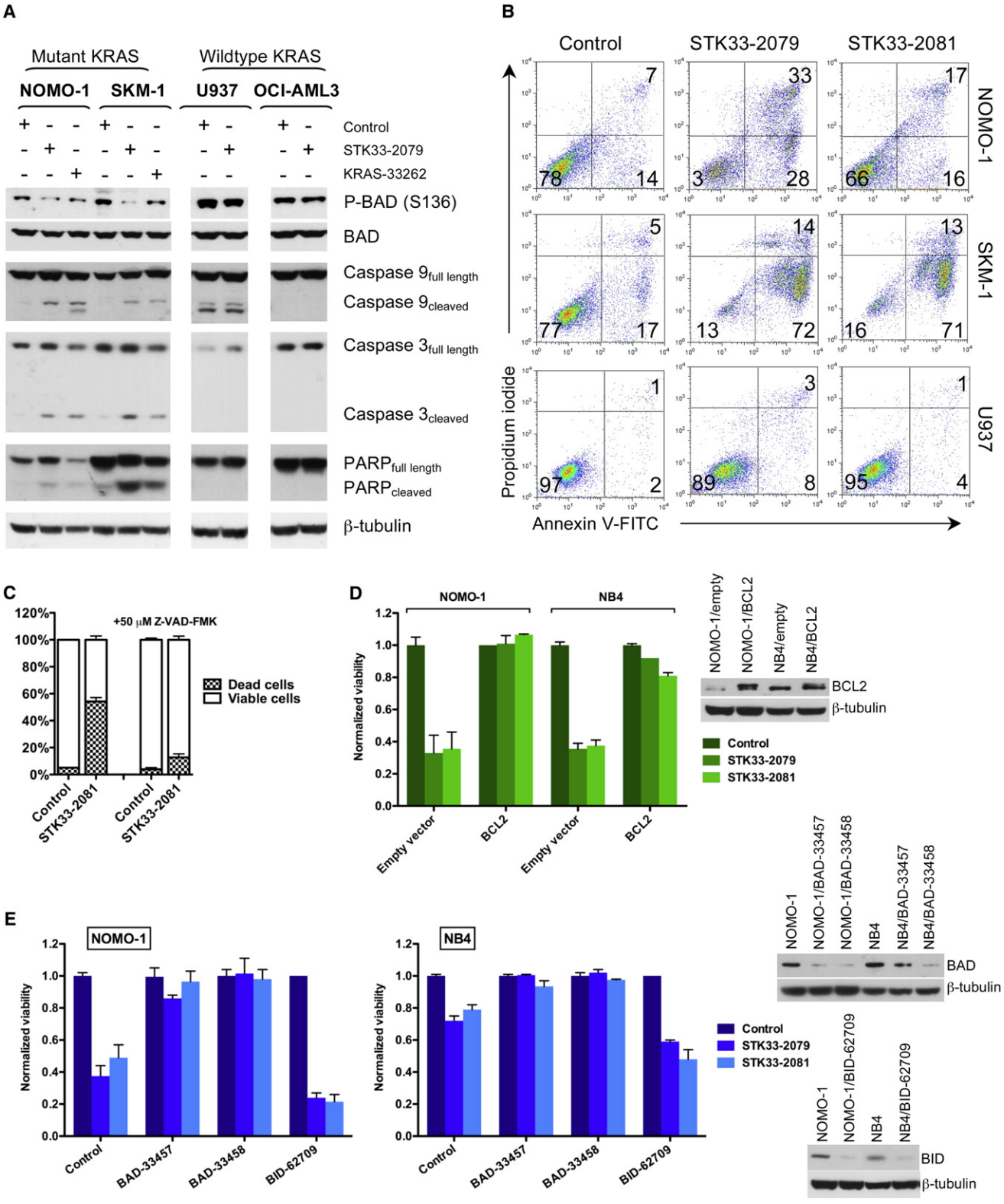


Figure 6. Selective Regulation of Mitochondrial Apoptosis by STK33 in Mutant *KRAS*-Dependent Cells

(A) Expression of proteins involved in apoptosis in AML cell lines transduced with shRNA constructs targeting *STK33* or *KRAS*.

(B) Apoptosis induction in AML cell lines transduced with shRNA constructs targeting *STK33*. Percentages of annexin V-positive/negative cells are indicated.

(C) Rescue of cell viability in NOMO-1 cells by incubation with Z-VAD-FMK prior to *STK33* knockdown.

cancer before, irrespective of tissue origin and genetic context. STK33 thus emerges as a component of a signaling pathway that is aberrantly required due to adaptation to a functionally relevant *KRAS* mutation.

Consistent with its role as a synthetic lethal interactor that is essential only in the context of mutant *KRAS* dependency, *STK33* expression alone does not appear to be sufficient for tumor initiation and maintenance, as reflected by the inability of *STK33* to transform murine cells in culture and the dispensability of *STK33* in human cancer cell lines that lack mutant *KRAS*. These observations are in agreement with the concept that many cancers are codependent on mutated oncogenes, which drive the malignant phenotype, as well as "normal" genes, a phenomenon that has been termed "nononcogene addiction" (Solimini et al., 2007). Recent studies have identified nononcogenes with specificity for particular cancer types, such as multiple myeloma, breast, and colon cancer (Schlabach et al., 2008; Shaffer et al., 2008; Silva et al., 2008), probably reflecting the distinct growth and survival requirements of different cell lineages. Our results indicate that dependencies on genes that have no transforming activity of their own can also be the consequence of individual cancer-causing mutations, such as oncogenic *KRAS* alleles, regardless of tissue context. The hypothesis that *STK33* does not act as a classical oncogene is further supported by the lack of structural abnormalities or deregulated expression of *STK33* in cancer cell lines and primary human cancer samples. This observation also illustrates the utility of RNAi screens for identifying candidate cancer drug targets that will evade detection by other genomic technologies.

The mechanism by which STK33 promotes cancer cell viability and proliferation involves genotype-selective regulation of S6K1 activity. Of particular interest in this context is the observation that STK33 enhances cell survival by modulating the suppression of mitochondrial apoptosis mediated via S6K1-induced inactivation of the death agonist BAD. Recent studies have shown that cancer cells harboring mutations of the RAS downstream effector BRAF are dependent on constitutive RAF-MEK-ERK signaling and display exquisite sensitivity to pharmacologic inhibition of the BRAF substrate MEK (Solit et al., 2006). The mechanism of cell killing by MEK inhibitors involves induction of the proapoptotic BH3-only protein BIM (Cragg et al., 2008), which is known to be negatively regulated by the MEK-ERK pathway (Ley et al., 2005). Despite the epistatic relationship between mutant *BRAF* and oncogenic *RAS* alleles in human cancers, inhibition of MEK-ERK signaling has significantly lower cytotoxic activity against cells harboring transforming *KRAS* mutations (Solit et al., 2006), indicating that oncogenic KRAS possesses additional prosurvival properties that need to be antagonized to achieve efficient tumor cell killing. In support of this hypothesis, we identified suppression of BAD via STK33-S6K1 signaling as a previously unrecognized mechanism whereby mutant *KRAS*-dependent cells evade apoptosis. These observations notwithstanding, we recognize that effects on other downstream effectors of S6K1 that regulate cancer cell growth and metabolism (DeBerardinis et al., 2008) may also contribute to the inhibitory effects of *STK33* suppression.

Aberrant S6K1 activity in tumors occurs primarily in response to stimulation of its upstream regulator mTORC1 (Guertin and Sabatini, 2007). For example, signals that inhibit the TSC2 tumor suppressor, and thus activate mTORC1, include hyperactivation of the PI3K-AKT and MAPK pathways that are characteristic of many cancers (Shaw and Cantley, 2006). The realization that deregulated mTORC1 activity plays an important role in tumorigenesis has prompted the development of mTORC1 inhibitors as anticancer drugs, and early clinical trials have demonstrated that these agents possess activity against certain tumor types (Faivre et al., 2006). However, several mechanisms of resistance, such as S6K1-dependent negative feedback loops, have been identified (Shaw and Cantley, 2006). We observed that in mutant *KRAS*-dependent cells, S6K1 activity is regulated by STK33, yet the available data suggest that this functional relationship does not involve mTORC1 or components of the PI3K-AKT and MAPK signaling pathways. Although further work will be required to gain a complete understanding of how STK33 interacts with other upstream effectors of S6K1, these findings nevertheless illustrate that cancer-associated genetic alterations, such as transforming *KRAS* mutations, may result in signaling routes that could serve as context-specific therapeutic targets.

Our efforts to perform synthetic lethality screens in cells harboring mutant *RAS* are not without precedent. Stockwell and colleagues used human fibroblast-derived cell lines engineered to express a transforming *HRAS* allele to screen large collections of small molecules and identified compounds that induced apoptosis preferentially in cells with activated RAS-RAF-MEK signaling (Yagoda et al., 2007). We found that such synthetic lethal interactions can also be identified in cancer cell lines that express endogenous *KRAS* mutations and thus more faithfully represent the functional consequences of these alleles as well as the signaling networks already present in the individual tumors where the mutations occurred. This notion is also supported by the previous identification of small interfering RNAs and chemical entities that are toxic to *KRAS* mutant DLD-1 colon cancer cells but not a congenic derivative of DLD-1 in which the mutant *KRAS* allele had been deleted (Sarthy et al., 2007; Torrance et al., 2001).

Our findings add to those of previous studies that used loss-of-function RNAi screens to identify genes that are selectively required in cell lines derived from breast and colon cancer, multiple myeloma, and DLBCL (Boehm et al., 2007; Firestein et al., 2008; Ngo et al., 2006; Schlabach et al., 2008; Shaffer et al., 2008; Silva et al., 2008). Specifically, the experimental strategy employed in this study—screening of cell lines representing multiple tumor types—revealed that *STK33* is essential for mutant *KRAS*-dependent cancer cells regardless of tissue origin. These observations illustrate that functional genetic approaches to cancer gene discovery have applicability beyond

(D) Rescue of cell viability in mutant *KRAS*-dependent AML cell lines by *BCL2* expression prior to *STK33* knockdown. BCL2 protein expression was confirmed by western blotting.

(E) Rescue of cell viability in mutant *KRAS*-dependent AML cell lines by suppression of *BAD* prior to *STK33* knockdown. Suppression of BAD and BID protein was confirmed by western blotting. Error bars in (C)–(E) represent the mean ±SEM of triplicate experiments.

the identification of cancer type-specific lethal genes. Indeed, our results indicate that *STK33* dependency is selectively conferred by oncogenic *KRAS*, even to the exclusion of mutant *NRAS* and *PTPN11* alleles that are also "undruggable." Expanding this experimental approach to an even broader array of cell lines and genes together with improved data analysis tools may identify additional allele- or pathway-specific genetic codependencies that could serve as therapeutic targets.

On the basis of the results reported here, which emphasize the complementarity between functional and structural cancer genomics, we envision STK33 inhibition as a strategy for therapeutic intervention in a broad spectrum of tumors associated with mutant *KRAS*. Since the synthetic lethal interaction between mutant *KRAS* and *STK33* suppression appears to be a specific attribute of cells that are functionally dependent on mutant *KRAS*, it will be important to devise methods for identifying oncogenic *KRAS* dependency in primary human tumors. Furthermore, pharmacologic inhibition of STK33 may not completely phenocopy *STK33* knockdown. Although our observations provide evidence that the kinase activity of STK33 is required for its effects on cancer cell viability, further work is necessary to determine whether loss of other, noncatalytic functions of STK33 also contributes to the toxicity of *STK33* suppression for mutant *KRAS*-dependent cells. Finally, efforts to develop STK33 inhibitors as anticancer agents will require studies to define the physiological role of *STK33*.

EXPERIMENTAL PROCEDURES

RNAi Screens

Cell lines were screened with a subset of the Broad Institute TRC shRNA Library with a previously described high-throughput platform (Moffat et al., 2006; Root et al., 2006). Detailed methods and analytical approaches for identifying genes that were selectively required in *KRAS* mutant cell lines are described in the Supplemental Experimental Procedures.

STK33 and *KRAS* Knockdown and Cell Viability Assays

The specificity of the screening results for *STK33* and *KRAS* was confirmed through evaluation of multiple shRNAs (Figure S1), and selected shRNAs were used for further experiments. Cells were transduced with pLKO.1puro lentiviral shRNA vectors from the TRC shRNA Library. Lentiviral particles were produced by cotransfection of 293T cells with pLKO.1 constructs and packaging plasmids pMD.G and pCMVR8.91. Transfections were carried out with FuGENE 6 (Roche Diagnostics), and virus was harvested 48 and 72 hr after transfection. Cells were incubated with lentiviral supernatants in the presence of 8 μg/ml Polybrene (American Bioanalytical) for 30 hr, and infected cells were selected with 2–10 μg/ml puromycin. After selection, cells were washed and replated in medium containing 0.5 μg/ml puromycin, and the number of viable cells was determined on five consecutive days with the CellTiter 96AQ$_{ueous}$ One Solution Proliferation Assay (Promega).

Anchorage Independence Assays

Cancer cell lines transduced with pLKO.1 constructs (5×10^3 to 2×10^4 cells) or NIH/3T3 cells transduced with pMSCVneo retroviral constructs (5×10^3 cells) were suspended in a top layer of RPMI-1640 containing 10% fetal calf serum (FCS) and 0.35% soft agar (Noble agar; Sigma-Aldrich) and plated on a bottom layer of RPMI-1640 containing 10% FCS and 0.5% soft agar in 35 mm dishes. After 3 to 6 weeks, colonies were stained with 0.005% crystal violet, counted microscopically, and photographed with a dissection microscope.

Tumorigenicity Assays

Cancer cell lines transduced with pLKO.1 constructs (1×10^7 cells) or NIH/3T3 cells transduced with pMSCV constructs (1×10^7 cells) were injected subcutaneously into the flanks of NOD/SCID mice (NOD/MrkBomTac-*Prkdc*^{scid}; Taconic) or *nude* mutant mice (NU/J *Foxn1*^{nu}; Jackson Laboratory). Tumor dimensions were measured twice weekly. Mice were sacrificed when tumors reached a diameter of 15 mm or after 5 weeks of monitoring. Animal experiments were performed in accordance with the Children's Hospital Boston Animal Care and Use Committee guidelines under the protocol number A07-03-042R.

Focus Formation Assays

NIH/3T3 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS. So that the effects of *KRAS* alleles and *STK33* on cell contact inhibition could be determined, 5×10^2 NIH/3T3 cells transduced with pMSCV contructs were seeded on a layer of 1×10^5 untransduced cells, and cell foci were stained with 0.005% crystal violet and counted after 2 weeks. For assessment of changes in cell morphology induced by *KRAS* and *STK33*, 1×10^5 transduced NIH/3T3 cells were plated in 35 mm dishes and photographed after 72 hr by phase contrast microscopy.

Cytokine Independence Assays

Parental BaF3 cells were maintained in RPMI-1640 supplemented with 10% FCS and 10% WEHI-conditioned medium as a source of interleukin-3 (IL-3). BaF3 cells transduced with pMSCV contructs were seeded at a density of 0.33×10^6/ml in IL-3-free medium, and the number of viable cells was determined daily by trypan blue exclusion.

DNA Sequence Analysis

Amplification of *KRAS* and *STK33* exons from genomic DNA was performed with previously published oligonucleotide primers (Wood et al., 2007), and amplification products were sequenced in both directions.

Immunoprecipitation and Western Blotting

Immunoprecipitation and western blotting were performed as described previously (Kim et al., 2002; Rocnik et al., 2006). Detailed methods and antibodies are provided in the Supplemental Experimental Procedures.

Protein Kinase Assays

FLAG-tagged STK33 was immunoprecipitated from 293T cells. For detection of STK33 autophosphorylation, [γ-^{32}P]-incorporation was determined. For detection of myelin basic protein (MBP) phosphorylation, the nonradioactive MAP Kinase Assay Kit (Millipore) was used. Detailed methods are provided in the Supplemental Experimental Procedures.

Apoptosis Detection

After transduction with shRNA constructs targeting *STK33* and selection with puromycin, viable cells were isolated by density gradient centrifugation and replated in medium without puromycin. The percentage of apoptotic cells after 4 days was determined by flow cytometry after staining with annexin V-fluorescein and propidium iodide (Annexin V-FITC Apoptosis Detection Kit I; BD Biosciences).

Rescue of Cell Viability after *STK33* Knockdown

NOMO-1 cells treated with 50 μM Z-VAD-FMK (Calbiochem) were transduced with shRNA constructs targeting *STK33*. After selection with puromycin, cells were washed and replated in medium containing 50 μM Z-VAD-FMK, and the percentages of dead and viable cells after 4 days were determined by trypan blue exclusion. NOMO-1 and NB4 cells expressing *BCL2* or shRNAs targeting *BAD* or *BID* were transduced with shRNA constructs targeting *STK33*. After 3 days of selection with puromycin (exogenous *BCL2* expression) or culture without puromycin (*BAD* and *BID* knockdown), cells were washed and replated, and the percentages of dead and viable cells after 4 days were determined by trypan blue exclusion.

ACCESSION NUMBERS

The microarray data set reported in this paper has been deposited in Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo) with the accession number GSE15151.

SUPPLEMENTAL DATA

Supplemental Data include Supplemental Experimental Procedures, 14 figures, and two tables and can be found with this article online at http://www.cell.com/supplemental/S0092-8674(09)00316-X.

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