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

Claudia Scholl,1,15 Stefan Fröhling,1,15 Ian F. Dunn,2,3,4,5,6 Anna C. Schinzel,3,4,5,6 David A. Barbie,3,4,5,6,7 So Young Kim,3,4,5,6 Serena J. Silver,6 Pablo Tamayo,6 Raymond C. Wadlow,7,8 Sridhar Ramaswamy,6,7,8,9 Konstanze Döhner,10 Lars Bullinger,10 Peter Sandy,11 Jesse S. Boehm,6 David E. Root,6 Tyler Jacks,6,11,12 William C. Hahn,1,3,4,5,6,* and D. Gary Gilliland1,3,6,9,13,14,15

1Department of Medicine
2Department of Neurosurgery
Brigham and Women’s Hospital, Harvard Medical School, Boston, MA 02115, USA
3Department of Medical Oncology
4Center for Cancer Genome Discovery
5Center for Cancer Systems Biology
Dana-Farber Cancer Institute, Harvard Medical School, Boston, MA 02115, USA
6Broad Institute of Harvard and Massachusetts Institute of Technology, Cambridge, MA 02142, USA
7Department of Medicine
8Center for Cancer Research and Center for Regenerative Medicine
Massachusetts General Hospital, Harvard Medical School, Boston, MA 02114, USA
9Harvard Stem Cell Institute, Harvard University, Cambridge, MA 02138, USA
10Department of Internal Medicine III, University Hospital of Ulm, 89081 Ulm, Germany
11David H. Koch Institute for Integrative Cancer Research and Department of Biology, Massachusetts Institute of Technology, Cambridge, MA 02139, USA
12Howard Hughes Medical Institute, Massachusetts Institute of Technology, Cambridge, MA 02139, USA
13Department of Stem Cell and Regenerative Biology, Harvard University, Cambridge, MA 02138, USA
14Howard Hughes Medical Institute, Harvard Medical School, Boston, MA 02115, USA
15These authors contributed equally to this work

*Correspondence: william_hahn@dfci.harvard.edu (W.C.H.), ggilliland@rics.bwh.harvard.edu (D.G.G.)
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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 NFκB 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 and immortalized human mammary epithelial cells (HMECs) RNAi screens in eight human cancer cell lines, representing proliferation, we performed high-throughput loss-of-function to identify genes that are essential for cancer cell 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.531A→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 KRAS family members may play a role in tumorigenesis. KRAS codon 18 mutations have been detected in patients with lung adenocarcinoma, colorectal adenoma, and adenocortical 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

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 suppression 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

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

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

[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.
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.
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-
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. 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
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/germline/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 overexpression 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
enrichment of components of the mitochondrial apoptotic pathway among the genes that were differentially regulated in response to STK33 suppression.

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 S10), 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.

(D) Normalized viability of NOMO-1 and NB4 cells transfected with empty vector, BCL2, STK33-2079, or STK33-2081.

(E) Western blot analysis of BAD and BID in NOMO-1 and NB4 cells transfected with empty vector, STK33-2079, or STK33-2081.
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 dependent 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 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 tumorogenesis 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 (Faire 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...
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 dependencies 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 96AQueous One Solution Proliferation Assay (Promega).

Anchorage Independence Assays
Cancer cell lines transduced with pLKO.1 constructs (5 x 10^3 to 2 x 10^4 cells) or NIH/3T3 cells transduced with pMSCVneo retroviral constructs (5 x 10^5 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 x 10^7 cells) or NIH/3T3 cells transduced with pMSCV constructs (1 x 10^7 cells) were injected subcutaneously into the flanks of NOD/SCID mice (NOD/MrkBomTac-Prdxextd, Taconic) or nude mutant mice (NU/J Foxn1nu; 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 x 10^5 NIH/3T3 cells transduced with pMSCV constructs were seeded on a layer of 1 x 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 x 10^4 transduced NIH3T3 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 constructs were seeded at a density of 0.33 x 10^5/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-32P]-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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