The p53 Target Gene SIVA Enables Non–Small Cell Lung Cancer Development

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Although p53 transcriptional activation potential is critical for its ability to suppress cancer, the specific target genes involved in tumor suppression remain unclear. SIVA is a p53 target gene essential for p53-dependent apoptosis, although it can also promote proliferation through inhibition of p53 in some settings. Thus, the role of SIVA in tumorigenesis remains unclear. Here, we seek to define the contribution of SIVA to tumorigenesis by generating Siva conditional knockout mice. Surprisingly, we find that SIVA loss inhibits non–small cell lung cancer (NSCLC) development, suggesting that SIVA facilitates tumorigenesis. Similarly, SIVA knockdown in mouse and human NSCLC cell lines decreases proliferation and transformation. Consistent with this protumorigenic role for SIVA, high-level SIVA expression correlates with reduced NSCLC patient survival. SIVA acts independently of p53 and, instead, stimulates mTOR signaling and metabolism in NSCLC cells. Thus, SIVA enables tumorigenesis in a p53-independent manner, revealing a potential new cancer therapy target.

SIGNIFICANCE: These findings collectively reveal a novel role for the p53 target gene SIVA both in regulating metabolism and in enabling tumorigenesis, independently of p53. Importantly, these studies further identify SIVA as a new prognostic marker and as a potential target for NSCLC cancer therapy.

See related commentary by Resnick-Silverman and Manfredi, p. 581.
The four exons comprising the locus (F1 and R2) were used. The absence of the floxed allele following Ad-Cre infection verifies the ability of Cre to fully excise the Siva allele. The 11-kb wild-type allele and the 8.5-kb band indicates the targeted conditional allele. Right, upon probing with the 5′ or 3′ probe, the 11-kb band indicates the wild-type allele and the 4.5-kb band indicates the targeted conditional allele. PCR analysis of recombined allele. MEFs generated from E13.5 Sivafl/− embryos (where fl denotes the conditional knockout allele) were infected either with adenovirus-expressing Cre (Ad-Cre) to excise the Siva allele or with empty adenovirus (Ad-Emp) as a control. Primers spanning the 5′loxP site (F1 and R1) or the remaining loxP site after excision of the Siva locus (F1 and R2) were used. The absence of the floxed allele following Ad-Cre infection verifies the ability of Cre to fully excise the Siva locus.

Figure 1. Generation of Siva conditional knockout mice. A, targeting scheme for generating Siva conditional knockout mice. The Siva-targeting vector contains a positive selection marker (Puromycin cassette [Puro]) flanked by loxP sites (triangles) and a negative selection marker (diphtheria toxin [dTA]). The four exons comprising the Siva locus (gray boxes) are flanked by loxP and Lox-Puro-Lox sites on the 5′ and 3′ ends, respectively. The Puro cassette was removed in vivo by limited Cre expression, leaving a single 3′loxP site. Upon subsequent Cre recombinase expression, the Siva locus gets excised, resulting in a Siva-null allele. These recombination events were detected by Southern blot analysis using XmnI/EcoR1 restriction digests and subsequent probing with a 5′ or 3′ fragment external to the targeting vector. This leads to generation of fragments of different sizes in all cases, as shown. B, BamHI; E1, EcoR1; XmnI, XmnI; Xh1, XhoI. B, Southern blot analyses of mouse embryonic stem (ES) cells targeted at the Siva locus. Analyses of a wild-type (Siva+/+) and a targeted (Sivafl+/−) ES cell clone are shown. DNA was digested with XmnI/EcoR1. Left, upon probing with the 3′ probe, the 11-kb band indicates the wild-type allele and the 8.7-kb band indicates the targeted conditional allele. Right, upon probing with the 5′ probe, the 8.5-kb band indicates the wild-type allele and the 4.5-kb band indicates the targeted conditional allele. C, PCR analysis of recombined allele. MEFs generated from E13.5 Sivafl/− embryos were infected either with adenovirus-expressing Cre (Ad-Cre) to excise the Siva allele or with empty adenovirus (Ad-Emp) as a control. Primers spanning the 5′loxP site (F1 and R1) or the remaining loxP site after excision of the Siva locus (F1 and R2) were used. The absence of the floxed allele following Ad-Cre infection verifies the ability of Cre to fully excise the Siva locus.

which p53 plays a key role in suppressing malignant progression. Using Siva conditional knockout mice that we generated, we found that SIVA is necessary for efficient oncogenic KRAS–driven NSCLC development. Subsequent analysis of SIVA function in vitro revealed that SIVA knockdown dampens proliferation and transformation in both mouse and human NSCLC cell lines. The diminished proliferation and transformation upon SIVA knockdown are independent of p53 and, instead, are associated with decreased metabolic function. These findings indicate that SIVA plays a p53-independent role in enabling lung cancer development and suggest the possibility that it could ultimately be a target for cancer therapy.

RESULTS

Generation of Siva Conditional Knockout Mice

To analyze the role of SIVA in tumorigenesis in vivo, we generated Siva conditional knockout mice. Mouse embryonic stem (ES) cells were targeted such that the entire Siva locus, comprising four exons, was flanked by a loxP and a Lox-Puro-Lox cassette, ensuring complete deletion of the Siva gene (Fig. 1A). Proper targeting of ES cells was confirmed by Southern blot analysis using both 5′ and 3′ external probes (Fig. 1B), and two independent lines of mice were generated. These mice were crossed to CMV-Cre transgenic mice (16), and progeny were screened for the presence of either a Siva conditional (denoted floxed or fl) or Siva-null allele. To confirm that the floxed Siva allele could be efficiently recombined to generate Siva-null cells, MEFs derived from E13.5 Sivafl/fl embryos were infected with adenovirus-expressing Cre recombinase (Ad-Cre), and DNA was harvested. PCR verified that the floxed Siva allele was efficiently recombined, thereby producing Siva-null cells (Fig. 1C). Furthermore, analysis of progeny from crosses between mice heterozygous for the Siva-null allele revealed embryonic lethality of Siva homozygous mutant animals, similar to that observed previously with mice homozygous for a Siva gene–trapped allele (S.B.R. Jacobs and J.L. Van Nostrand, unpublished data; data not shown).
SIVA Enables KRAS-Induced Lung Tumorigenesis

To determine the role of SIVA in tumorigenesis, we chose an NSCLC model in which p53 has been shown to play a critical role in suppressing tumor progression (17). In this model, activated KRAS<sup>G12D</sup> drives the development of lung adenomas, which progress to adenocarcinomas in the absence of p53 (5, 17). We used <i>Kras<sup>LSL-G12D</sup></i> mice, which we infected with Ad-Cre through intratracheal injection (18) to drive expression of KRAS<sup>G12D</sup> and excision of the <i>Siva</i> locus. Mice were subjected 18 weeks later to a quantitative analysis of tumor development (Fig. 2A). Surprisingly, histologic analyses revealed fewer tumors, including both adenomas and adenocarcinomas, and reduced tumor burden (percentage of lung area comprising tumors) in <i>Kras<sup>LSL-G12D, Siva<sup>fl/−</sup></sup></i> mice compared with <i>Kras<sup>LSL-G12D,Siva<sup>+/+</sup></sup></i> control mice. <i>Kras<sup>LSL-G12D,Siva<sup>fl/−</sup></sup></i> mice also had reduced burden of hyperplastic lesions compared with controls (Fig. 2B–E). In addition, we observed reduced tumor number and tumor burden in <i>Siva</i> heterozygous mice (<i>Kras<sup>LSL-G12D,Siva<sup>fl/+</sup></sup></i>, <i>Kras<sup>LSL-G12D,Siva<sup>fl/+</sup></sup></i>) relative to <i>Kras<sup>LSL-G12D,Siva<sup>fl/−</sup></sup></i> controls (Supplementary Fig. S1A and S1B). Furthermore, the tumor burden and tumor number in <i>Kras<sup>LSL-G12D,Siva<sup>−/−</sup></sup></i>–heterozygous mice were comparable with those observed in <i>Kras<sup>LSL-G12D,Siva<sup>fl/−</sup></sup></i> mice. Together, these observations indicate that SIVA loss impedes tumor initiation, and that SIVA normally enables tumorigenesis.

**Figure 2.** Siva deficiency inhibits lung tumorigenesis. A, schematic of the timeline for tumor study. Six- to 8-week-old mice were infected with Ad-Cre via intratracheal injection, and lungs were analyzed 18 weeks later. B, representative photomicrographs of lungs from Kras<sup>LSL-G12D,Siva<sup>fl/−</sup></sup> (left, n = 12) and Kras<sup>LSL-G12D,Siva<sup>fl/+</sup></sup> (right, n = 17) mice 18 weeks after Ad-Cre infection by intratracheal injection. C, box plot depicting the median number and quartiles of hyperplasias and tumors (adenomas and adenocarcinomas) per total lung area in Kras<sup>LSL-G12D,Siva<sup>fl/−</sup></sup> and Kras<sup>LSL-G12D,Siva<sup>fl/+</sup></sup> mice. Dots represent outlier data points. Hyperplasias: ***, P = 0.008; tumors: ***, P = 0.0058 by the Wilcoxon rank sum test between Kras<sup>LSL-G12D,Siva<sup>fl/−</sup></sup> and Kras<sup>LSL-G12D,Siva<sup>fl/+</sup></sup> mice. D, box plot depicting median tumor burden and quartiles calculated as tumor area per total lung area for hyperplasias and tumors (adenomas and adenocarcinomas). Dots represent outlier data points. Hyperplasia: *, P = 0.006; tumors: ***, P = 0.003 by Wilcoxon rank-sum test between Kras<sup>LSL-G12D,Siva<sup>fl/−</sup></sup> and Kras<sup>LSL-G12D,Siva<sup>fl/+</sup></sup> mice. E, representative photomicrographs of hyperplasias and tumors (adenomas and adenocarcinomas) taken at ×100 and ×400 magnification.
Siva Knockdown Inhibits Mouse NSCLC Cell Proliferation

To explore the mechanisms underlying the decreased tumorigenesis observed following SIVA inactivation, we used two mouse NSCLC cell lines, LSZ2 and LSZ4, generated previously by serial allograft passage of tumors derived from Kras^{G12D};Tpr{+} mice. Importantly, these tumor cells still harbor wild-type p53 (19) and express SIVA (Fig. 3A). We used two independent shRNAs directed against Siva and achieved a 70% to 80% reduction in SIVA protein levels following lentiviral transduction with each (Fig. 3B), indicating that SIVA facilitates proliferation. In contrast, depleting Siva by infecting Siva^{EG} MEFs with Ad-Cre or knocking down Siva in pancreatic cancer cell lines did not affect the proliferative index (Supplementary Fig. S2A and S2B), suggesting that SIVA’s role in facilitating proliferation is cell type specific or context specific. Notably, Siva knockdown in LSZ4 cells did not augment the percentage of Annexin V-positive cells, indicating that increased apoptosis is not responsible for diminished tumor burden (Supplementary Fig. S3A and S3B). Next, to more directly explore whether Siva knockdown affects the transformation properties of NSCLC cells, we assessed the ability of cells to form colonies upon low-density plating and upon anchorage-independent growth in soft agar.

Siva knockdown resulted in fewer colonies forming in the low-density plating assay compared with shGFP control-expressing cells (Fig. 3C). Similarly, using a soft-agar assay, fewer colonies were able to form upon Siva knockdown than with shLacZ control shRNA (Fig. 3D). Together, these results suggest that SIVA enhances proliferation and transformation, thus recapitulating our findings in the in vivo tumorigenesis study.

SIVA Knockdown Inhibits Human NSCLC Cell Proliferation

To determine whether the role for SIVA in promoting efficient tumorigenesis in mouse cells is conserved in human cells, we next examined whether SIVA similarly facilitates the proliferation of human A549 NSCLC cells expressing oncogenic KRAS^{G12D} and wild-type p53. We knocked down Siva in A549 cells using lentiviral transduction of SIVA shRNAs and observed depletion of SIVA mRNA by quantitative RT-PCR (Fig. 4A). We assessed the importance of SIVA for cellular proliferation by generating growth curves and measuring BrdUrd incorporation. As observed in the mouse LSZ4 and LSZ2 cell lines, Siva knockdown in A549 cells results in reduced proliferative potential relative to the shGFP control (Fig. 4B and C). Furthermore, knockdown of SIVA inhibited colony formation in both low-density plating and soft-agar assays relative to the shGFP control, revealing an important role for SIVA in promoting transformation (Fig. 4D and E). These results demonstrate that SIVA enhances proliferation and transformation not only in mouse NSCLC cells but also in human
SIVA is Necessary for Tumorigenesis

NSCLC cells. Supporting the idea that SIVA expression can promote tumorigenesis, analysis of survival data for human NSCLC patients revealed that patients with lung cancers expressing high levels of SIVA have significantly reduced overall survival than those with lung cancers expressing low levels of SIVA (Fig. 4F; refs. 20, 21). Thus, SIVA expression levels have prognostic power in human NSCLC patients.

Siva Knockdown Inhibits Proliferation and Transformation in a p53-Independent Manner

Decreased proliferative capacity and suppression of transformation ability are well-known effects of p53 activation. It has previously been reported that SIVA can negatively regulate p53 by stabilizing the p53–MDM2 interaction and by promoting p19ARF degradation and that SIVA loss can therefore induce p53 activity (14, 15). We therefore sought to test whether stabilization and activation of p53 provokes the inhibition of proliferation and transformation we observed upon SIVA inactivation in NSCLC cells. First, we assessed p53 protein levels and activity upon Siva knockdown in LSZ4 cells. Western blot analysis of p53 in LSZ4 cells after Siva knockdown revealed no difference in p53 levels, despite a clear inhibition of proliferation (Fig. 5A). Second, because p53 is a transcriptional activator, we analyzed p53 target gene expression as a readout of p53 activity. Upon Siva knockdown in LSZ4 cells, we observed no difference in p53 target gene expression relative to shGFP-expressing cells, suggesting that SIVA does not stimulate proliferation in NSCLC cell lines by inactivating p53, as previously described (Fig. 5B). Finally, we investigated whether p53 is required for the phenotypic effects of Siva knockdown. Upon attenuation of both Trp53 and Siva in LSZ4 cells using RNA interference, we assessed BrdUrd incorporation and found that additional knockdown of Trp53 does not rescue the inhibition of proliferation triggered by Siva loss (Fig. 5C–E). These results were bolstered by analysis of Trp53-null NSCLC cell lines, where we observed that Siva knockdown also causes decreased transformation in soft-agar assays, similarly to Siva knockdown in p53-expressing LSZ4 cells (Fig. 5F). Thus, the inhibition of proliferation caused by attenuated Siva expression does not rely on p53.

Siva Knockdown Alters Expression of Metabolic Genes

To gain an understanding of the molecular basis for the reduced proliferation and transformation resulting from Siva knockdown, we performed gene expression profiling experiments on LSZ4 and LSZ2 cells after Siva knockdown by lentiviral transduction with each of the two previously characterized shRNAs (shSiva1 and shSiva2) and each of two control shRNAs (shLacZ and shGFP). Hierarchical clustering revealed a notable
difference in gene expression profiles between Siva knockdown and control samples (Fig. 6A). Differentially expressed genes were analyzed for enrichment in Gene Ontology (GO) terms using GeneSpring Analysis. We found that the most enriched GO terms included immune regulation—consistent terms using GeneSpring Analysis. We found that the most genes were analyzed for enrichment in Gene Ontology (GO) down and control samples (Fig. 6A). Differentially expressed

Siva

showed to impinge upon NF-

knockdown in LSZ4 cells. ACTIN was used as a loading control. B, qRT-PCR analysis of p53 target gene expression, after normalization to β-Actin, in NSCLC cells transduced with shSiva or shGFp. Error bars, ±SD. n = 2. C, Western blot analysis of p53 and SIVA upon knockdown of Trp53 and/or Siva in LSZ4 cells. ACTIN was used as a loading control. D, average of percentage of BrdUrd incorporation in LSZ4 cells upon knockdown of Trp53 and/or Siva, shGFp and shLuc served as controls. Error bars, ±SD. n = 3. E, phase-contrast images of LSZ4 cells upon knockdown of Trp53 and/or Siva. F, left, images of colony formation in soft-agar assays performed in Trp53-null NSCLC cells with knockdown of Siva. Right, average number of colonies formed relative to shLuc-transduced cells in soft-agar assays. Error bars, ±SD; n = 3. **, P = 0.01.

Siva Knockdown Decreases Metabolic Activity and Induces Autophagy

To interrogate the role of SIVA in regulating metabolism, we first examined whether metabolic function is altered upon Siva knockdown by measuring oxygen consumption rates (OCR) and extracellular acidification rates (ECAR), as indicators of oxidative phosphorylation and glycolytic rates, respectively. These analyses revealed that knockdown of Siva in both LSZ4 and LSZ2 cells decreases mitochondrial respiration and, less dramatically, glycolysis, suggesting an overall reduction in metabolic function (Fig. 6C and data not shown). Specifically, although the basal oxidative phosphorylation rate was reduced slightly upon Siva knockdown, the maximal mitochondrial respiration capacity, measured upon treatment with the ATP synthesis–electron transport uncoupler FCCP, was significantly decreased upon Siva knockdown. Treatment with either oligomycin, an ATP synthase inhibitor that reveals the OCR associated with mitochondrial proton leak, or antimycin, a mitochondrial complex III inhibitor that disrupts the proton gradient and reveals mitochondrial-independent oxygen consumption, did not cause any significant change in OCR upon Siva inhibition, suggesting a specific role for SIVA in mitochondrial metabolism. To investigate why mitochondrial function is decreased upon Siva knockdown, we examined mitochondrial content by quantifying mitochondrial DNA, specifically the mitochondrial Cox3, CybB, and 16S rRNA genes. Quantitative PCR revealed that mitochondrial DNA content is reduced by approximately 40% in both LSZ2 and LSZ4 cells upon Siva knockdown, providing a basis for the decreased mitochondrial respiration function (Fig. 6D). These results suggest that SIVA is critical for promoting efficient metabolism through the mitochondria, and thus SIVA may normally promote proliferation by facilitating efficacious ATP production.
An important homeostatic response of cells to decreased energy production is to induce autophagy, a process by which organelles and cytoplasmic proteins are degraded for either energy production or quality control. We therefore sought to query whether autophagy might be induced upon Siva knockdown. We assessed levels of autophagy by immunoblotting for modified LC3 (LC3-II), which is generated from LC3-I upon conjugation of phosphatidylethanolamine and then incorporated into autophagc vesicles, thus serving as a marker of autophagy. To confirm that any enhanced LC3-II signal represents increased autophagic flux, rather than a block in autophagy resulting in the accumulation of
SIVA loss decreases metabolic function of NSCLC cells. A, gene set enrichment analysis (GSEA) of genes with altered expression upon Siva shRNA transduction into LSZ2 and LSZ4 cells relative to cells with control shRNA transduction. The FDR P value and normalized enrichment score (NES) for each category are shown on the right side. The GSEA signature for decreased mTOR signaling is highlighted in gray. B, select GSEA profiles (left) enrichment of genes upregulated upon mTOR inhibition in the presence of AKT upregulation. FDR q-value: 0.089 (right) enrichment of genes down-regulated upon mTOR activation. FDR q-value: 0.069. C, Western blot analysis of mTOR signaling targets in LSZ4 cells upon shSiva or shGFP control transduction. ACTIN serves as a loading control. D, average percentage of BrdUrd incorporation in LSZ4 cells treated with 50 nmol/L Torin1 or 50 nmol/L rapamycin for 18 hours. Error bars, ±SD; *P value by the Student's t test. E, Torin1: 0.048, **, rapamycin: 0.009. F, Western blot analysis of mTOR upstream signaling components in LSZ4 cells upon shSiva or shGFP control transduction. ACTIN serves as a loading control. F, average percentage of BrdUrd incorporation in LSZ4 cells with control (shLacZ) or Siva (shSiva) knockdown and without (siControl) or with TSC2 (siTSC2) knockdown. Error bars, ±SD. *, P < 0.05; **, P < 0.01 by the Student's t test. G, model depicting the role of SIVA in promoting tumorigenesis through activation of mTOR signaling, which can itself affect metabolism, proliferation, tumorigenesis, and autophagy. SIVA may also have mTOR-independent effects on these processes.
mTOR, which also has a known role in regulating mitochondrial metabolism, inhibiting autophagy, promoting cell proliferation, and supporting tumorigenesis (27). These observations, coupled with our finding that Siva knockdown produces a signature resembling those associated with decreased mTOR signaling, suggest that SIVA may promote mTOR signaling (Fig. 7B). To test this hypothesis, we first examined whether Siva knockdown results in decreased mTOR activity by analyzing phosphorylation of the mTOR substrates S6 kinase (S6K) and 4EBP1 as well as the S6K substrate S6. Indeed, we observed a reduction in phospho-S6K, phospho-S6, and phospho-4EBP1 levels upon Siva knockdown, indicative of diminished mTOR activity, consistent with the gene expression data (Fig. 7C). These findings indicate that SIVA is indeed necessary for full mTOR activity. We next examined whether reduced mTOR activity results in diminished proliferation in LSZ4 cells. We used two mTOR inhibitors, Torin1 and rapamycin, which target mTOR complex 1 and both mTOR complexes 1 and 2, respectively, and quantified the rate of BrdUrd incorporation. Similar to Siva knockdown, inhibition of mTOR activity significantly decreased the proliferation of LSZ4 cells, suggesting that mTOR activity is indeed necessary for maximal LSZ4 cell proliferation (Fig. 7D).

mTOR is regulated either by AMPK, which inhibits mTOR activity through phosphorylation and inhibition of RAPTOR at S722/S792 and phosphorylation and activation of TSC2 at S1387, or by mitogenic signaling pathways, which activate mTOR by phosphorylating and inhibiting TSC2 at S1462 (27). To determine how SIVA inhibits mTOR activity, we examined the phosphorylation of RAPTOR at S792, as a marker of AMPK signaling, and phosphorylation of TSC2 at S1462, as a marker of mitogenic signaling pathways. Although we observed no difference in phospho-RAPTOR levels in cells with different Siva status, we observed a decrease in phospho-S1462 TSC2 levels upon Siva knockdown, suggesting that SIVA acts through mitogenic signaling rather than AMPK signaling to promote mTOR activity (Fig. 7E). Together, these findings reveal that SIVA is essential for maximal mTOR activity, which is necessary for optimal proliferation in LSZ4 cells.

To determine whether decreased mTOR activity is responsible for the proliferation defects upon Siva knockdown, we tested whether reactivation of mTOR signaling through knockdown of Tsc2 could rescue BrdUrd incorporation in shSiva-transduced cells. Indeed, with approximately 50% knockdown of Tsc2, we observed a partial rescue in BrdUrd incorporation in shSiva-transduced cells and no effect in control cells (Fig. 7F). Collectively, these findings reveal a novel role for SIVA in promoting mTOR activity and metabolism, which is necessary for enhanced proliferation and tumorigenesis downstream of oncogenic KRAS (Fig. 7G).

**DISCUSSION**

Here, we describe a critical role for the p53 target gene SIVA in facilitating NSCLC development. Conditional inactivation of Siva in an autochthonous oncogenic KRASG12D-driven model for NSCLC resulted in decreased tumor numbers and tumor burden relative to SIVA-expressing controls. Moreover, we found that SIVA knockdown inhibits proliferation and transformation in both mouse and human NSCLC cells in vitro. We discovered further that SIVA does not exert its protumorigenic effect by restraining p53. Instead, the enhancement of tumorigenesis by SIVA relates to its ability to promote mTOR signaling, inhibit autophagy, and augment metabolic activity (28–30). Consistent with this protumorigenic role, high-level SIVA expression correlates with worse prognosis in NSCLC patients (20).

Previous reports showed that SIVA loss promotes p53 stabilization and inhibits proliferation, attributable to SIVA function in enabling p53-MDM2 interactions and in enhancing ubiquitin-mediated p19ARF degradation (14, 15). In contrast, we found that p53 activation is not responsible for the inhibition of proliferation upon SIVA loss, as p53 was not induced upon Siva attenuation and Siva knockdown could inhibit proliferation and transformation irrespective of p53 status. However, the observation that SIVA is able to promote tumorigenesis reveals an intriguing contradiction with respect to its role as a p53 target gene, perhaps reflecting the fact that p53 target genes are often regulated by additional transcription factors and function in a p53-independent manner in some contexts. For example, SIVA can also be induced by the E2F1 transcription factor, which can play both tumor-suppressive and oncogenic roles (12, 31). Such a protumorigenic role of a p53 target gene recalls TIGAR, a p53 target gene involved in cell survival upon oxidative stress, which promotes tumorigenesis in a mouse small-intestinal cancer model (32). Moreover, TIGAR is thought to be upregulated in a p53-independent manner in human tumors (33). Our work similarly suggests that SIVA plays a p53-independent role in promoting proliferation and tumorigenesis.

Although SIVA clearly alters the metabolic capacity of NSCLC cells, reflected by enhanced mitochondrial respiration and reduced autophagy, it remains unclear precisely how SIVA exerts these effects. SIVA may affect metabolism by stimulating mTOR signaling, which is known to enhance oxidative metabolism, at least in part, via activation of S6K and 4EBP1 and consequent translation of nuclear-encoded mitochondrial proteins (34, 35), and to dampen autophagy through phosphorylation and inactivation of the autophagy-initiating kinase ULK1/ATG1 (36). Moreover, mTOR is known to promote tumorigenesis in mouse hepatocellular, renal, and lung cancer models (28, 29, 37, 38). Thus, a role for SIVA in the positive regulation of mTOR could account for the decreased oxidative phosphorylation, increased autophagy, and reduced tumor burden with SIVA deficiency (27, 34, 36, 38). Based on our Western blot analyses of upstream signaling pathway components, SIVA appears to exert effects on mTOR signaling not through the negative regulator AMPK, which responds to cellular energy levels, but rather through positively regulating mitogenic signaling pathways, such as the ERK1/2, WNT, and AKT signaling pathways, which respond to growth signals (27, 37). However, the exact mechanisms by which SIVA activates these mitogenic signaling pathways and which mitogenic signaling pathways are most important remain to be elucidated.

An alternative mechanism for SIVA action is that it affects metabolism through pathways related to its apoptotic function.
A precedent for this notion comes from the observation that loss of both BAX and BAK, which renders cells completely apoptosis deficient, triggers increased autophagy and decreased mTOR signaling in response to stress signals, as a measure to maintain cell viability (39, 40). Loss of SIVA could similarly promote autophagy in the presence of a stress signal such as oncogenic KRAS. That SIVA loss could mimic BAX/BAK deficiency is consistent with the observation that BAX and BAK are required for SIVA to induce apoptosis (11). A role for SIVA at the mitochondria is further supported by the decreased mitochondrial function observed upon SIVA knockdown. Interestingly, mitochondrial respiration has been found to be required for mitochondrial apoptosis by allowing for efficient mitochondrial depolarization and release of proapoptotic factors (41, 42). Thus, diminished mitochondrial respiration may be a mechanism by which SIVA deficiency protects cells from apoptosis, in keeping with the important role for SIVA in apoptosis (11). Overall, elucidating the mechanisms through which SIVA deficiency inhibits mTOR signaling, dampens oxidative phosphorylation, and triggers autophagy will be an important future goal for understanding how SIVA promotes tumorigenesis.

In addition to defining a role for SIVA in efficient mTOR signaling, we also identified additional signaling pathways affected by SIVA status in our microarray experiments. One particularly interesting signaling pathway upregulated upon SIVA knockdown is the TBK1 pathway. TBK1 is an IkB kinase that promotes survival through the NFkB and AKT pathways and is required for KRAS-dependent NSCLC proliferation. (26, 43). However, TBK1 has also been shown to inhibit mTOR activity and promote autophagy (44). Thus, although upregulation of TBK1 signaling could explain how SIVA deficiency inhibits mTOR activity and drives autophagy, the proliferative activity attributed to activated TBK1 contrasts with the decreased proliferation observed upon SIVA inactivation, suggesting that TBK1 is not a primary SIVA mediator. Another intriguing signature associated with SIVA knockdown is activated HIPPO-YAP signaling. The YAP pathway controls organ size and tumorigenesis by promoting cell proliferation and inhibiting apoptosis (45). Moreover, YAP activates the P13K-mTOR pathway by inhibiting PTEN (37, 46). Therefore, it is possible that SIVA activates the mTOR pathway by activating the YAP pathway. Another signature identified upon SIVA knockdown is that of inhibition of BM11, a component of the polycomb repressive complex, which promotes both cell self-renewal and tumorigenesis (47). Although loss of BM11 results in decreased tumorigenic potential and decreased mitochondrial oxidative capacity, like SIVA loss, it is not known to promote other phenotypes associated with SIVA deficiency, such as inhibition of mTOR signaling (48). It will be of great interest in future studies to further evaluate the role of SIVA in these additional signaling pathways to better define the mechanisms by which SIVA promotes tumorigenesis.

Our finding that SIVA is necessary for efficient tumorigenesis in an autochthonous lung cancer model suggests the possibility of SIVA as a therapeutic target. Indeed, the protumorigenic role of SIVA is supported by a previous xenograft study in which SIVA knockdown in U2OS cells was found to inhibit tumor growth relative to control knockdown cells (14).

Furthermore, our finding that alternate cell types, including primary MEFs, are not adversely affected by SIVA loss suggests that SIVA inhibition could represent a useful strategy for cancer treatment. In future studies, it will also be interesting to further elaborate the role of SIVA in vivo using mouse models for other cancer types to determine whether SIVA generally plays an enabling role in tumorigenesis or if SIVA might play context-dependent roles in cancer development, sometimes suppressing tumor development. Regardless, the discovery of a role for SIVA in enhancing tumorigenesis unveils a potential new therapeutic target for cancer treatment.

**METHODS**

**Generation of Siva Conditional Knockout Mice**

The Siva locus was isolated from a BAC clone, 122D14, obtained from the Sanger Institute (Cambridge, UK), using BspE1 and BstE11 restriction enzymes. The fragment including Siva was modified to incorporate a 5′ LoxP site at the BspE1 site and a 3′ Lox-PGK-Puro-Lox cassette at the BstE11 site and subcloned into the 184-DT-PL vector. The Siva targeting construct was introduced into J1 ES cells by electroporation. ES cells were cultured on irradiated MEF feeders in LIF-containing media (Millipore). Positive clones were identified by PCR and Southern blot analysis for both 5′ and 3′ targeting and used to generate chimeric mice at the Stanford transgenic research facility. Chimeras were bred to C57BL/6 females and progeny genotyped by Southern blot analysis or PCR as described below. Mice were further bred to CMV-Cre mice to excise the Puro cassette, resulting in a single LoxP site 3′ of the Siva locus (Sivα). For analysis, Siva conditional mice were bred to KrasG12D;G12D mice. All animal work was done in accordance with the Stanford University (Stanford, CA) Administrative Panel on Laboratory Animal Care.

**Genotyping**

Genotyping was performed by PCR analysis of tail DNA. The primers used include a forward primer 5′ for the LoxP site (F1: AGT ACC AGC ATT CCC TGG TG), a reverse primer 3′ for the LoxP site (R1: GGA GTC AGA CCT CGT TAC GG), and a reverse primer 3′ for the LoxPuroLox site (R2: CCA CAC AGG ACA TTC GAC AG). For analysis, Siva conditional mice were bred to KrasG12D;G12D mice. All animal work was done in accordance with the Stanford University (Stanford, CA) Administrative Panel on Laboratory Animal Care.

**Lung Tumor Analysis**

For the tumor study, mice were intratracheally injected with 4 × 10⁶ plaque-forming units of Ad-Cre [University of Iowa Gene Transfer Vector Core (GTVC), Iowa City, IA], as described (18). Mice were sacrificed 18 weeks later for analysis of tumor number and burden. Lungs were formaldehyde-fixed, each lobe bisected or trisected, and paraffin-embedded. Histologic analysis was performed on hematoxylin and eosin–stained sections. Lung area and tumor area were quantified using ImageJ.

**Cell Culture**

LSZ2, LSZ4, A549, and Trp53-null NSCLC cells and MEFs were cultured in DMEM with 10% serum (3, 19). LSZ2 and LSZ4 cells were a gift from Alejandro Sweet-Cordero (Stanford School of Medicine, Stanford, CA). Trp53 status was confirmed by Western blot analysis and target gene expression analyses, but cell lines were not otherwise authenticated. Trp53-null NSCLC cells were derived from KrasG12D;Trp53-null mice as described previously (3). MEFs were derived from wild-type or Siva−/− E13.5 embryos, and genotypes were determined by PCR. Cells were last tested for Mycoplasma using Lonza MycoAlert Assay (LT07-118) at the time of revisions. Adenoviral infections were performed at a multiplicity of infection of approximately 100 using adenoviral Cre or empty (Ad-Cre or Ad-Emp; University of Iowa GTVC) for
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24 hours. Cells were imaged using phase-contrast microscopy. For BrdUrd incorporation analysis, cells were incubated with 3 μg/mL BrdUrd for 4 hours before fixation of cells on coverslips using 4% paraformaldehyde. Cells were treated with 50 mM L-ramipycin, 50 mM/L Torin1, or 100 mM/L Chloroquine for 18 hours prior to the BrdUrd pulse. Tsc2 was knocked down with Dharmaco smart pool siRNAs (cat. no. #L-047050-00) using Lipofectamine RNAiMax reagent (Life Technologies), and cells were BrdUrd-pulsed 72 hours later. Immunofluorescence was performed as described with anti-BrdUrd antibody (1:50, 347580; BD Biosciences; ref. 5). Proliferation was monitored using a Sulforhodamine B assay as described (49) on trichloroacetic acid-fixed cells collected every 2 days over a 7-day period. For low plating assays, 150 cells were plated into 6-well dishes and collected 10 days later, and colonies were stained with crystal violet. For soft-agar assays, 105 cells were cultured in 2.4% noble agar in DMEM with 10% serum or for 2 or 3 weeks. Colonies were visualized after staining with 0.02% Giemsa and quantified using ImageJ on scanned images. For respiration rate analysis, 10^4 cells were visualized after staining with 0.02% Giemsa and quantified using ImageJ on scanned images. For respiration rate analysis, 10^4 cells were plated in each well of a 96-well plate and analyzed for OCR and ECAR on an XF96 Extracellular Flux Analyzer (Seahorse Biosciences) upon treatment with oligomycin, FCCP, or antimycin. Rates were normalized to cell number.

Western Blot Analysis

Cells were collected and lysed with RIPA buffer (1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, Tris pH 8.0) with protease inhibitors (Complete; Roche) or SDS buffer (20% SDS, Tris pH 6.8) with protease and phosphatase inhibitors (Roche). Western blot analyses were probed with anti-p53 (1:500, CMS; Vector Labs), anti-IL-3 (1:2000, NB100-2220; Novus), anti-S6K (1:1,000, 9206; Cell Signaling Technology), anti-pS6K (1:1,000, 2708; Cell Signaling Technology), anti-β2-microglobulin . For qPCR

Microarray Analysis

Microarray analysis was performed on RNA isolated by TRIzol extraction and reverse transcribed using Moloney murine leukemia virus reverse transcriptase (Invitrogen) and random primers, and PCR was performed in triplicate using SYBR green (Qagen, Bio-Rad) and specific primers for each gene (Supplementary Table S1) in a 7900HT Fast Real-Time PCR machine (Applied Biosystems). Results were computed relative to a standard curve made with cDNA pooled from all samples and normalized to β-actin.

qPCR

For qRT-PCR, NSCLC cells were cultured at subconfl uency, RNA was isolated by TRIzol extraction and reverse transcribed using Moloney murine leukemia virus reverse transcriptase (Invitrogen) and random primers, and PCR was performed in triplicate using SYBR green (Qagen, Bio-Rad) and specific primers for each gene (Supplementary Table S1) in a 7900HT Fast Real-Time PCR machine (Applied Biosystems). Results were computed relative to a standard curve made with cDNA pooled from all samples and normalized to β-actin. For qPCR of mitochondrial DNA, NSCLC cells were cultured at subconfl uency, cells were lysed in RIPA buffer with Proteinase K, and DNA was purified with phenol/chloroform extraction followed by ethanol precipitation. PCR was performed in triplicate using SYBR green (Qagen, Bio-Rad) and mouse-specific primers for each mitochondrial gene. Results were computed relative to a standard curve made with DNA pooled from all samples and normalized to β2-microglobulin.

RESULTS

Microarray analysis was performed on RNA isolated by TRIzol extraction from LSZ2 and LSZ4 cells transfected with two independent ShRNA constructs (shSiva1 and shSiva2) or two control shRNAs (shGFP and shLaZ). RNA was hybridized to Agilent Sureprint G3 Mouse GE 8 X 60 K microarrays (G4852A) by the Stanford Functional Genomics Facility (microarray data files were deposited at Gene Expression Omnibus: GSE66126) and data were normalized and analyzed using GeneSpring GX software (Agilent). GO annotation was performed using GeneSpring GX software, and GSEA was performed using GSEA software (50).

Disclosures of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Conception and design: J.L. Van Nostrand, S.B.R. Jacobs, L.D. Attardi

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J.L. Van Nostrand, A. Brisac

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): J.L. Van Nostrand, A. Brisac, S.S. Mello, R. Luong, L.D. Attardi

Writing, review, and/or revision of the manuscript: J.L. Van Nostrand, A. Brisac, S.S. Mello, S.B.R. Jacobs, R. Luong, L.D. Attardi

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): J.L. Van Nostrand, R. Luong

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The p53 Target Gene SIVA Enables Non–Small Cell Lung Cancer Development

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