Genetic disruption of oncogenic Kras sensitizes lung cancer cells to Fas receptor-mediated apoptosis

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Genetic lesions that activate KRAS account for ∼30% of the 1.6 million annual cases of lung cancer. Despite clinical need, KRAS is still undruggable using traditional small-molecule drugs/inhibitors. When oncogenic *Kras* is suppressed by RNA interference, tumors initially regress but eventually recur and proliferate despite suppression of *Kras*. Here, we show that tumor cells can survive knockout of oncogenic *Kras*, indicating the existence of *Kras*-independent survival pathways. Thus, even if clinical KRAS inhibitors were available, resistance would remain an obstacle to treatment. *Kras*-independent cancer cells exhibit decreased colony formation in vitro but retain the ability to form tumors in mice. Comparing the transcriptomes of oncogenic *Kras* cells and *Kras* knockout cells, we identified 603 genes that were specifically up-regulated in *Kras* knockout cells, including the *Fas* gene, which encodes a cell surface death receptor involved in physiological regulation of apoptosis. Antibodies recognizing Fas receptor efficiently induced apoptosis of *Kras* knockout cells but not oncogenic *Kras*-expressing cells. Increased Fas expression in Kras knockout cells was attributed to decreased association of repressive epigenetic marks at the *Fas* promoter. Concordant with this observation, treating oncogenic Kras cells with histone deacetylase inhibitor and Fas-activating antibody efficiently induced apoptosis, thus bypassing the need to inhibit Kras. Our results suggest that activation of Fas could be exploited as an Achilles' heel in tumors initiated by oncogenic Kras.

Kras | lung cancer | Fas | apoptosis

Lung cancer is a leading cause of cancer death, accounting for ∼1.3 million deaths worldwide each year (1). Non–small-cell lung cancer (NSCLC), the most common subtype, is associated with frequent mutations in *KRAS* (∼30%). *KRAS* is also frequently mutated in other tumor types, including pancreatic (>90%) and colon (∼30%) cancer (2). Although various pharmacological inhibitors are being developed for RAS, especially for the mutant $KRAS^{G12C}$ (3–5), these small molecules have not been tested in the clinic (6, 7). As a result, advanced oncogenic KRAS lung cancers are usually treated with conventional therapy such as radiation and chemotherapy, often with limited success (1, 8).

Controlled expression of oncogenic RAS cDNA in mouse models of melanoma, lung, breast, and pancreatic cancer has shown that the withdrawal of oncogenic RAS results in complete tumor regression (9–11). This phenomenon, known as "oncogene addiction," suggests that oncogenic *KRAS* alleles (e.g., $KRAS^{G12D}$) not only initiate tumorigenesis but also play a crucial role in tumor maintenance. To recapitulate KRAS oncogene addiction in a mouse model of lung cancer, we developed a conditional *Kras* shRNA system (shKras) to knock down *Kras* in $Kras^{G12D/+}$;$p53^{-/-}$ (KP) cell lines derived from a mouse lung tumor (12–14). When we orthotopically transplanted shKras KP cells into immunocompromised mice, we found that Kras-driven lung tumors can escape oncogene addiction and become independent of Kras signaling (termed Kras independence) (15, 16).

Because shRNAs targeting Kras do not completely eliminate Kras expression, residual Kras in cells could contribute to Kras-independent tumor growth. The best way to rule out this possibility is to genetically delete *Kras* altogether. Unfortunately, the *Kras* knockout mouse is embryonically lethal (17), and genetic disruption of *KRAS*—or other oncogenes—in human cells remains a challenge due to the low efficiency of homologous recombination using traditional gene-targeting technology. We recently showed that CRISPR (18) can be used to efficiently and specifically edit cancer genes in adult mice in a fraction of the time and cost of traditional mouse models (19–21). CRISPR therefore provides a flexible genetic system to manipulate the function of cancer genes (22, 23).

Previous work has shown that oncogenic KRAS epigenetically silences Fas expression (24, 25). In addition, RAS directs epigenetic silencing of *Fas* through a highly ordered pathway that culminates in methylation of the *Fas* promoter (26, 27). It remains unexplored whether Fas can be restored by genetic inactivation of oncogenic RAS.

Significance

Oncogenic *KRAS* underlies 30–90% of lung, colon, and pancreatic cancers, but despite more than 30 y of research, clinical inhibitors of KRAS—and potential resistance mechanisms—remain elusive. Using CRISPR-mediated genome editing of oncogenic *Kras*, we show that some lung cancer cells can survive *Kras* knockout, indicating the existence of mechanisms that allow tumors to escape Kras oncogene addiction. We identify genes highly expressed in Kras knockout cells, including the Fas receptor gene. Antibodies that activate Fas receptor selectively induced apoptosis in Kras-independent lung cancer cells, suggesting a potential strategy for combinatorial therapies against Kras-driven tumors. These findings have direct translational implications for the treatment of lung cancer and other KRAS mutant cancer types.

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Data deposition: The deep-sequencing data reported in this paper have been deposited in the National Center for Biotechnology Information BioProject database (accession no. PRJNA356881).

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Here, we use CRISPR to establish viable *Kras* knockout ($Kras^{-/-}$) lung cancer cell lines from parental oncogenic *Kras* ($Kras^{G12D/+}$) cells. While investigating the mechanism of Kras-independent tumorigenesis in this model, we identified *Fas* among the genes most highly regulated by Kras. Fas (also known as CD95, APO-1, and TNFRSF6) encodes a cell surface death receptor that triggers apoptosis upon binding by its cognate ligand, Fas ligand (FasL) (or CD95L), and plays critical roles in the immune elimination of cancer cells (28, 29). In both mouse and human lung cancer cells, genetic disruption of Kras elevated Fas expression on the cell surface and increased sensitivity to Fas-mediated apoptosis, thereby demonstrating a selective vulnerability of Kras-independent cells. Consistent with previous work showing that oncogenic KRAS epigenetically silences Fas expression (24, 25), we show that Fas is activated in $Kras^{-/-}$ cells by loss of both Dnmt1 and Ezh2 recruitment and repressive epigenetic marks of the *Fas* promoter. Remarkably, treatment of parental $Kras^{G12D/+}$ cells with pharmacological histone deacetylase (HDAC) inhibitors not only increased Fas levels but also sensitized cells to Fas-mediated apoptosis. These results suggest a combinatorial strategies for targeted elimination of Kras-independent and oncogenic Kras lung cancer cells.

Results

***Kras* Knockout Murine Lung Cancer Cells Are Viable and Can Form Tumors in Mice.** Our previous study showed that shRNAs targeting Kras do not completely eliminate Kras in cells (16), hence the residual Kras might contribute to Kras independence. We therefore used CRISPR-based method to genetically disrupt oncogenic Kras in two independent mouse $Kras^{G12D/+}$;$p53^{-/-}$ lung cancer cell lines (termed KP1 and KP2) (30–32). Lentiviral vector (lentiCRISPR) with puromycin selection marker, as described previously (33), was used to deliver Cas9 and a sgRNA targeting $Kras^{G12D}$ (sgKras) into the target cells (Fig. 1*A*). The puromycin-resistant single-cell clones expressing sgKras were screened for Kras elimination and specific indel mutations at target loci. Immunoblot analysis of single clones expressing sgKras, using anti-Kras antibody showed the total absence of endogenous Kras protein (Fig. 1*B*). Furthermore, deep-sequencing analysis revealed 2-nt deletions at the G12D allele (Fig. 1*C*). Although we designed sgKras to target the G12D sequence (Fig. 1*A*), a 1-nt deletion was detected at the wild-type allele (Fig. 1*C*). This confirmed that CRISPR can tolerate mismatch between the sgRNA and target site, consistent with known CRISPR off-target effects (34). These biallelic deletions shift the reading frame of *Kras* mRNA, likely resulting in premature termination of translation and nonsense-mediated decay of the *Kras* mRNA, as shown in immunoblots of Fig. 1*B*. We therefore defined these clones as $Kras^{-/-}$ (Kras knockout) cells.

The $Kras^{-/-}$ clones show markedly reduced proliferation and colony-forming ability compared with $Kras^{G12D/+}$ (Fig. 1*D*), further validating the role of *Kras* in cell proliferation in lung cancer. Nevertheless, $Kras^{-/-}$ cells are viable and form small colonies in an in vitro colony formation assay (Fig. 1*D*).

To test whether $Kras^{-/-}$ cells can form tumors in vivo, we transplanted $Kras^{G12D/+}$ and $Kras^{-/-}$ cells s.c. in immunosuppressed nude mice. $Kras^{-/-}$ cells formed tumors ($n = 3$ mice), but detection of tumor was much slower (60 d) compared with $Kras^{G12D/+}$ cells (20 d) (Fig. S1*A*). In addition, we observed gland-like structure and more cytoplasm of $Kras^{-/-}$ tumor by H&E staining (Fig. S1*B*), providing availability to further investigate $Kras^{-/-}$ tumor pathology. Taken together, these data show that cancer cells derived from oncogenic *Kras* tumors can indeed escape complete genetic disruption of *Kras*, and the resulting Kras-independent cancer cells can still form tumors in nude mice.

Transcriptome Analysis of Kras Knockout Cells Revealed Distinct Gene Signatures. To explore the mechanisms of Kras independence, RNA sequencing (RNA-seq) was performed in $Kras^{G12D/+}$ and $Kras^{-/-}$ cells. Bioinformatic analysis of RNA-seq data identified

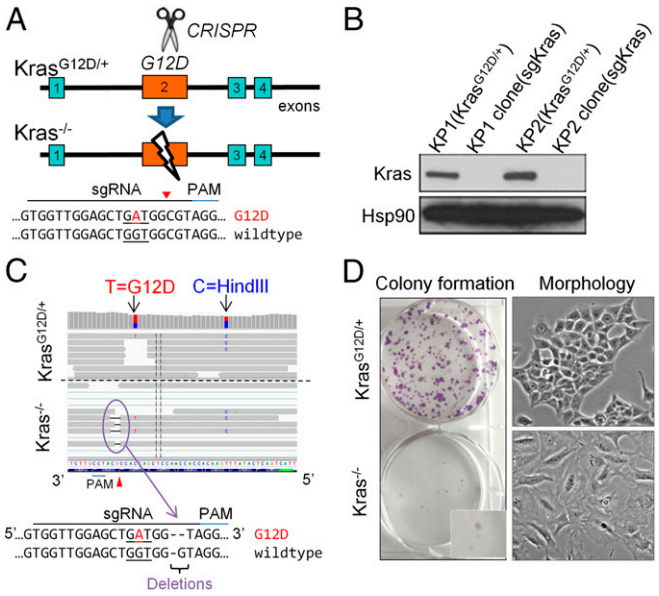

Fig. 1. CRISPR-mediated *Kras* knockout in Kras-driven mouse lung adenocarcinoma cells. (*A*) Schematic diagram of CRISPR sgRNA design targeting exon 2 of the mutant *Kras* allele ($Kras^{G12D/+}$). Codon 12 is underlined. "GAT" encodes G12D mutation. Red arrowhead indicates the Cas9 cutting site. (*B*) Immunoblot demonstrating total Kras protein levels in KP1 and KP2 clone pairs. Each pair included two clones: one parental (KP1/KP2) and one *Kras* knockout (KP1 clone/KP2 clone). Hsp90 was used as a loading control. (*C*) Deep-sequencing analysis showing small deletions induced by CRISPR in a representative clone. (*Upper*) Representative IGV plots. Black bars denote deletions (purple circle). The red "T" is the G12D mutation. The HindIII site ("C" SNP) was engineered in the original $Kras^{G12D/+}$ mouse model. (*Lower*) CRISPR induced a 2-nt deletion in the $Kras^{G12D}$ allele and a 1-nt deletion in the wild-type *Kras* allele. (*D*) Colony formation assay to examine the ability of $Kras^{G12D/+}$ and $Kras^{-/-}$ cells to form colonies. Cells were seeded in six-well plates at 1,000 cells per well, cultured for 5 d and stained with crystal violet. *Inset* shows small $Kras^{-/-}$ colonies.

603 up-regulated genes and 216 down-regulated genes in $Kras^{-/-}$ compared with $Kras^{G12D/+}$ cells (absolute fold change, >2; value of $P < 0.05$; Fig. 2 *A* and *B*), which were consistently up-regulated (down-regulated) in both pairs of $Kras^{G12D/+}$ and $Kras^{-/-}$ cells (KP1 and KP2, Fig. 2 *A* and *B*). These genes are listed in Dataset S1 ("DEG differentially expressed genes"). We then analyzed whether these differentially expressed genes were enriched in any gene ontology or pathways, using the Gene Set Enrichment Analysis (GSEA) and the Molecular Signature Database (MSigDB) (35). The 603 up-regulated genes were enriched in many cancer-related pathways (Fig. 2*C*), most notably epithelial to mesenchymal transition (q value = 7.25E-53), YAP (q value = 8.30E-18) as well as sets of genes that are down-regulated upon activation of an oncogenic form of KRAS (q value = 1.08E-29). Conversely, the 216 down-regulated genes are enriched in the sets of genes up-regulated by KRAS activation (q value = 3.27E-9). The entire list of enriched gene sets and pathways are provided in Dataset S1 ("Panther or GSEA"). We further confirmed that mRNA levels of RAS ortholog genes (*Hras* and *Nras*) did not change in $Kras^{-/-}$ cells (Fig. 2*D*). In addition, the sgKras did not induce indel mutations at *Hras* and *Nras* genomic loci (Fig. S2 *A* and *B*). These data suggest that Hras and Nras do not compensate for the inactivation of oncogenic Kras in this model.

To narrow down the list of differentially expressed genes and identify a possible secondary target in $Kras^{-/-}$ cells, we made the following assumptions: (*i*) the gene should be up-regulated upon Kras knockout, (*ii*) the gene should encode a plasma membrane protein, allowing it to be more easily targeted. Out of the

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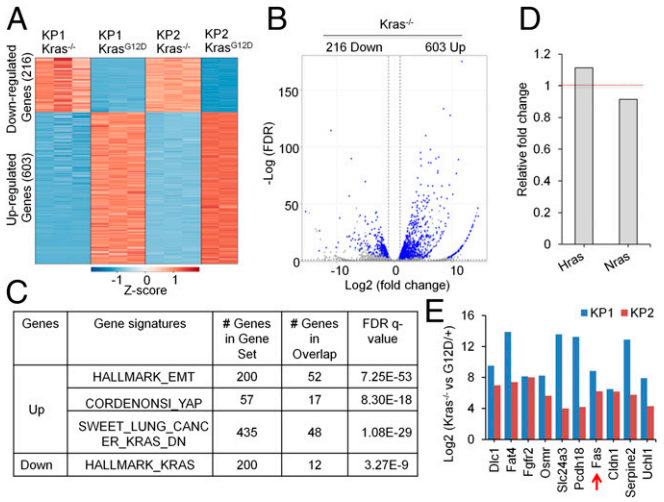

Fig. 2. Identification of KRAS independence genes by RNA-seq. (*A*) RNA-seq in $Kras^{G12D/+}$ and $Kras^{-/-}$ cell pairs to identify Kras-independent genes. Heat map shows the clustering of differentially expressed genes. $n = 2$ for KP2 $Kras^{-/-}$, $n = 3$ for other groups. (*B*) Volcano plot of differentially expressed genes in $Kras^{-/-}$ vs. $Kras^{G12D}$ cells. A total of 603 genes showed increased expression in both KP1 and KP2 pairs; 216 genes showed decreased expression. (*C*) Selected GSEA dataset. GSEA analysis identified gene sets enriched in up-regulated genes (up in $Kras^{-/-}$ cells) or in down-regulated genes (down in $Kras^{-/-}$ cells). (*D*) RNA-seq reads showing the relative expression of Ras family genes *Hras* and *Nras* in $Kras^{-/-}$ cells. (*E*) Top 10 candidate genes in the "membrane protein" category.

603 up-regulated genes, 189 were annotated as plasma membrane proteins (Dataset S1). To identify up-regulated genes with the most significant *P* values and largest fold changes, we ranked each of the 189 genes by its average rank of *P* value. Fold change of the top 10 genes in $Kras^{-/-}$ clones is shown in Fig. 2*E*. We further analyzed the expression profile of these top 10 candidate genes using The Cancer Genome Atlas (TCGA) datasets (lung adenocarcinoma vs. normal), and found that 7 candidate genes were significantly down-regulated in tumor samples (Fig. S2*C*). This analysis indicated that these candidates may have a putative role in development or progression of human lung cancer. Moreover, some of these of the candidates, such as DLC1 (36), FAT4 (37), and FAS (28), are well-known tumor suppressors.

Fas Is Up-Regulated in Kras-Independent Cancer Cells. Among the top 10 genes highly expressed in $Kras^{-/-}$ cells from the RNA-seq, we focused to investigate Fas (which encodes the Fas receptor) for two reasons. First, the Fas receptor triggers apoptosis through cell-intrinsic pathway (38, 39), and it has been implicated in various malignancies (28), hence an attractive therapeutic target. Second, based on previous work (26, 27), Fas expression has been shown to be regulated by Ras-dependent pathways. Following validation of RNA-seq data, the quantitative RT-PCR (qRT-PCR) and immunoblot analysis in parental $Kras^{G12D/+}$ and $Kras^{-/-}$ cells revealed that $Kras^{G12D/+}$ cells expressed very low levels of Fas, whereas $Kras^{-/-}$ cells showed a marked increase in Fas mRNA and protein levels (Fig. 3 *A* and *B*). Oncogenic RAS activates several downstream signaling pathways, including the MAPK and phosphoinositide 3-kinase (PI3K)/AKT pathways (2). Consistent with previous reports, that Kras-dependent silencing of *Fas* is partly mediated by MAPK pathway (26), we also observed an increased level of Fas mRNA in $Kras^{G12D/+}$ cells treated with MEK inhibitor U0126 (Fig. 3*C*).

Previous studies have shown that expression of oncogenic RAS in mouse NIH 3T3 cells transcriptionally silenced Fas, thereby preventing Fas ligand-induced apoptosis (25). Subsequent studies using genome-wide RNAi screens identified cofactors required for RAS-mediated epigenetic silencing of Fas (27). Kras directs epigenetic silencing of Fas through an ordered pathway that culminates in methylation of the Fas promoter and recruitment of corepressor complex (26, 27). Because $Kras^{G12D/+}$ cells expressed relatively lower levels of Fas mRNA, we hypothesized that Fas could be transcriptionally silenced in these cells. The Kras-mediated epigenetic silencing of *Fas* requires two major events, first is the methylation of promoter by DNA methyltransferase, DNMT1, and then trimethylation of histone H3 at lysine 27 (H3K27me3) catalyzed by the histone methyltransferase Ezh2, a component of polycomb repressive complex 2 (26, 27). shRNA-mediated knockdown of either Ezh2 or Dnmt1 significantly increased Fas expression in $Kras^{G12D/+}$ cells, suggesting that both factors mediate Kras-dependent silencing of Fas (Fig. S3*A*).

We then measured the levels of Dnmt1 and EZH2 and their corresponding epigenetic marks at the Fas promoter in $Kras^{G12D/+}$ and $Kras^{-/-}$ cells. Chromatin immunoprecipitation (ChIP) revealed a significant enrichment of EZH2 and H3K27me3 at the *Fas* promoter in $Kras^{G12D/+}$ cells compared with $Kras^{-/-}$ cells (Fig. 3 *D* and *E*). Consistent with previous studies (27), Dnmt1 and DNA methylation were also significantly enriched at the Fas promoter in $Kras^{G12D/+}$ cells compared with $Kras^{-/-}$ cells (Fig. 3 *F* and *G*). Thus, the high levels of Fas mRNA in $Kras^{-/-}$ cells is consistent with low levels of Dnmt1 and EZH2 and their corresponding epigenetic marks at the Fas promoter. Moreover, we found that H3K4 acetylation was enriched at the *Fas* promoter in $Kras^{-/-}$ cells (Fig. S3*B*), consistent with its transcriptionally active state. These data indicate that genetic inactivation of oncogenic Kras leads to transcriptional activation and restoration of Fas expression.

Fas-Mediated Apoptosis Can Eliminate Kras-Independent Cells. Because Fas is a major mediator of apoptosis on the cell surface, we hypothesized that activation of Fas might selectively trigger apoptosis of $Kras^{-/-}$ cells. Using fluorescence-activated cell sorting (FACS), we confirmed that the Fas receptor is highly expressed on

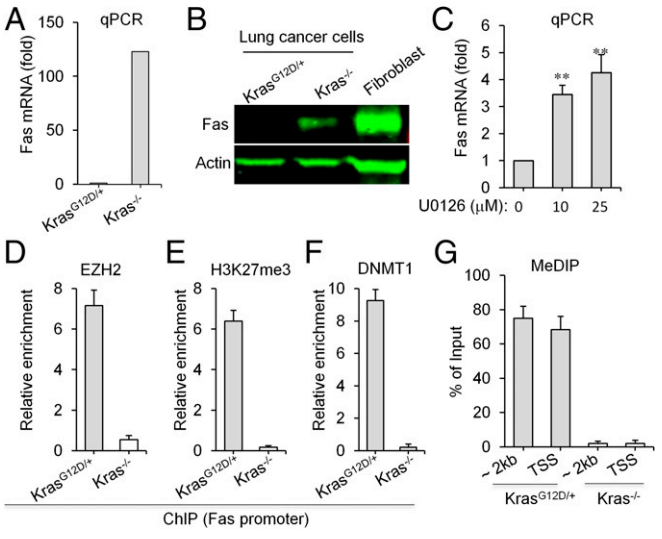

Fig. 3. The *Fas* receptor is up-regulated in $Kras^{-/-}$ cells. (*A*) qPCR showing up-regulation of *Fas* mRNA in $Kras^{-/-}$ cells. (*B*) Immunoblot demonstrating Fas protein levels in $Kras^{G12D/+}$ *and* $Kras^{-/-}$ cells. Mouse 3T3 fibroblast cells were used as a positive control for Fas expression. (*C*) qPCR showing an increase in *Fas* mRNA upon treatment of MEK inhibitor U0126 in $Kras^{G12D/+}$ cells at 48 h. (*D–F*) ChIP assays demonstrating the relative binding of EZH2, DNMT1, and enrichment of H3K27Me3 on Fas promoter in $Kras^{G12D/+}$ and $Kras^{-/-}$ cells. (*G*) Methylated DNA immunoprecipitation (MeDIP) assays demonstrating the relative enrichment of 5-methyl cytosine on Fas promoter in $Kras^{G12D/+}$ and $Kras^{-/-}$ cells. Error bars represent SD ($n = 3$). $^{**}P < 0.01$.

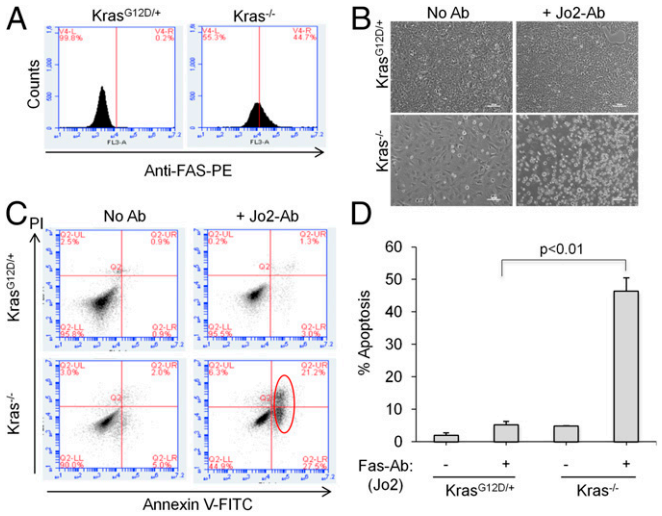

Fig. 4. *Kras* knockout mouse NSCLC cells are sensitive to Fas-mediated apoptosis. (*A*) FACS histogram showing the Fas receptor expression in $Kras^{-/-}$ and $Kras^{G12D/+}$ cells. (*B*) Fas-activating antibodies (Jo2) induces apoptosis in $Kras^{-/-}$ but not $Kras^{G12D/+}$ cells. Cells were incubated with Jo2 for 24 h. Bright-field images show floating apoptotic $Kras^{-/-}$ cells (*Lower Right*). (*C*) Dot plot showing the percentage of Annexin V- or propidium iodide (PI)-positive $Kras^{-/-}$ and $Kras^{G12D/+}$ cells. Red circle denotes Annexin V-positive cells. *x* and *y* axes denote Annexin V and PI signals. (*D*) Quantitation of Annexin V-positive cells represented as percentage of apoptotic cells. Error bars are SD ($n = 3$).

the surface of $Kras^{-/-}$ cells compared with $Kras^{G12D/+}$ cells (Fig. 4*A*). As reported before, binding of Fas ligand or Fas-activating antibodies to Fas receptor triggers apoptosis through cell-intrinsic pathway (28). We tested whether a Fas-activating antibody can induce Fas-mediated cell death in $Kras^{-/-}$ cells. When incubating cells with an antibody (Jo2), which binds and activates mouse Fas receptor (28), we observed that many $Kras^{-/-}$ cells became round in shape and detached from the plate, compared with few dead $Kras^{G12D/+}$ cells (Fig. 4*B*). Furthermore, we measured the apoptotic cells by Annexin V staining using FACS and observed that ∼50% of Jo2-treated $Kras^{-/-}$ cells were Annexin V positive compared with ∼5% of Jo2-treated $Kras^{G12D/+}$ cells (Fig. 4 *C* and *D*), demonstrating that Fas activation can selectively induce apoptosis in $Kras^{-/-}$ cells over the oncogenic $Kras^{G12D/+}$ cells. These results demonstrated that, in the absence of Kras, Fas expression is significantly restored, conferring sensitivity to Fas antibody-induced apoptosis.

To investigate whether the Fas restoration can be rescued by oncogenic RAS, we reintroduced an oncogenic RAS cDNA in $Kras^{-/-}$ cells. Because $Kras^{-/-}$ cells constitutively expressed Cas9 and Kras sgRNA, these cells are refractory to a $Kras^{G12D}$ cDNA. However, our deep-sequencing data showed that the *Hras* allele is not affected by the Kras sgRNA. We introduced oncogenic HRAS in $Kras^{-/-}$ cells through retrovirus expressing a HRAS-V12 cDNA (40). Consistent with our previous results of Fas restoration upon loss of Kras (Fig. 3), overexpression of HRAS-V12 in $Kras^{-/-}$ cells led to dramatic decrease in Fas mRNAs (Fig. S4*A*). Moreover, Fas receptor-mediated apoptosis triggered by the Jo2 antibody was fully rescued in $Kras^{-/-}$ cells expressing HRAS-V12 (Fig. S4*B*). Thus, reintroduction of oncogenic RAS rapidly suppresses Fas expression in nearly all $Kras^{-/-}$ cells. Together, our data suggest Fas activating antibody as a potential therapeutic strategy to kill Kras-independent cancer cells following *Kras* silencing.

KRAS Knockout Human Cancer Cells Are Sensitive to FAS-Mediated Apoptosis. To assess whether CRISPR can edit *KRAS* in human NSCLC cells carrying *KRAS* mutations, we chose A549 human lung adenocarcinoma cells, which harbor a homozygous $KRAS^{G12S}$ allele (41). Using similar approach as described above, A549 cells were infected with lentivirus expressing Cas9 and a guide RNA that targets human *KRAS* (sgKRAS). sgKRAS induced genome editing (Fig. S5) and significantly reduced total KRAS protein level in a cell population (Fig. 5*A*). Concordant with our results in mouse lung cancer cells, we found that some A549 cells survive *KRAS* knockout. Likewise, we also observed an increase in FAS mRNA (Fig. 5*B*) and receptor level (Fig. 5*C*) in sgKRAS-expressing A549 cells.

To explore methods to overcome resistance to KRAS knockout in human cells, we hypothesized that activation of FAS could also selectively induce apoptosis in *KRAS* knockout A549 cells. By incubating cells with an activating antibody for human FAS (clone EOS9.1), we observed that *KRAS* knockout cells are sensitive to FAS-mediated apoptosis (Fig. 5 *D*–*F*). These results indicate a potential strategy to overcome resistance for KRAS inhibition in human NSCLC by activating FAS.

To ensure that the increased sensitivity to FAS activation is indeed a phenotype of CRISPR-mediated KRAS knockout, we used previously reported DLD1 colon cancer cells ($KRAS^{G13D/+}$ vs. $KRAS^{-/+}$) generated by targeting G13D allele by traditional homologous recombination (42). As expected, $KRAS^{-/+}$ DLD1 cells also exhibited increased sensitivity to FAS antibody-induced apoptosis. As shown in Fig. 6 *A* and *B* and Fig. S6, incubation of FAS-activating antibody with KRAS knockout ($KRAS^{-/+}$) DLD1 cells, markedly increased Annexin V-positive apoptotic cells. This suggests that *KRAS* knockout leading to restored FAS expression is a general phenomenon, and that *KRAS* silencing confers sensitivity to FAS-induced apoptosis both in lung cancer cells edited by CRISPR and in colon cancer cells generated by traditional homologous recombination. Therefore, this conserved mechanism may provide a therapeutic strategy to suppress tumor relapse alongside future generation of KRAS inhibitors (Fig. 6*C*).

A Combination of HDAC Inhibitor and Fas Antibody Induces Apoptosis in Oncogenic Kras Cells. DNA methylation on CpG motifs in gene promoters is often accompanied by the recruitment of HDACs to the transcriptional regulatory site, thereby altering local chromatin structure and inhibiting transcription. General inhibitors of class I and II HDACs, alone or in combination with the methylation

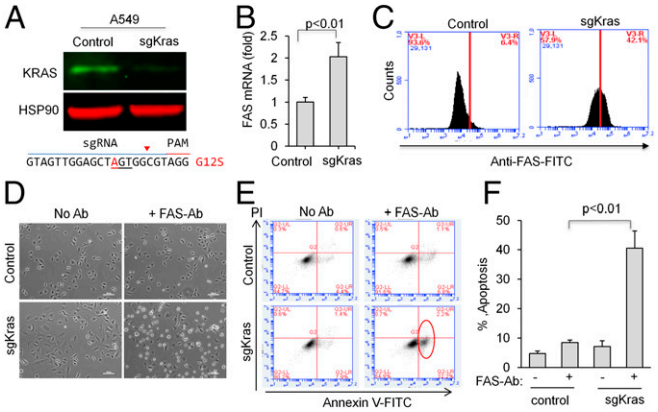

Fig. 5. *KRAS* knockout human NSCLC cells are sensitive to FAS-mediated apoptosis. (*A*) CRISPR-mediated *KRAS* editing in human NSCLC cell line A549 with a homozygous G12S mutation. Pooled cells expressing sgKRAS were analyzed by immunoblot to detect total KRAS protein level. HSP90 was used as a loading control. (*B*) qPCR measurement of FAS mRNA in control and sgRNA-expressing cells. (*C*) FACS histogram showing the levels of human FAS receptor in A549 control or sgKRAS cells. (*D*) Representative bright-field images showing floating apoptotic cells, in the presence of an activating antibody recognizing human FAS receptor. (*E*) Dot plot showing percentage of Annexin V- or PI-positive cells. Red circle denotes Annexin V-positive cells. (*F*) Quantification of *E*. Error bars represent SD ($n = 3$).

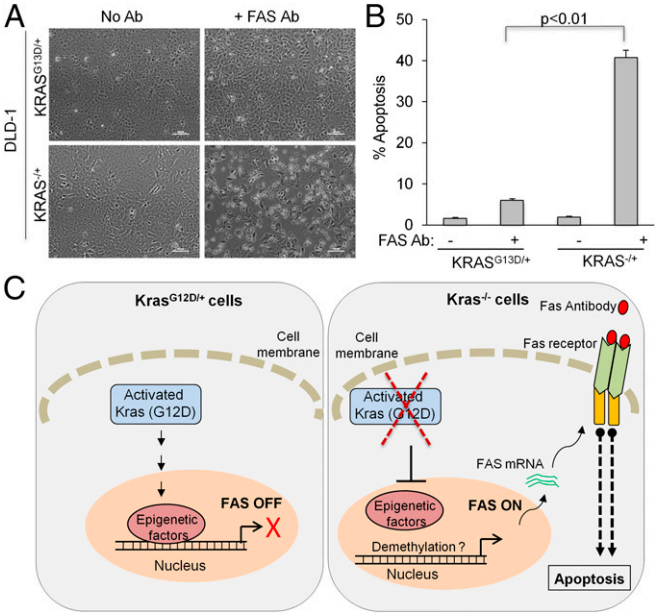

Fig. 6. KRAS knockout DLD1 cells are sensitive to FAS-mediated apoptosis. (*A*) KRAS knockout (KRAS$^{-/+}$) and parent KRAS$^{G13D/+}$ DLD1 cells were incubated with an activating antibody against human FAS. Representative bright-field images show floating/apoptotic KRAS$^{-/+}$ cells. (*B*) Quantitation of apoptosis measured by Annexin V/PI staining and FACS analysis. Error bars are SD (n = 3). (*C*) A simplified model of how KRAS silencing may increase FAS expression and sensitize cells to FAS receptor-mediated apoptosis.

inhibitor 5-azacitydine, or its congener 5-aza-deoxycytidine (5Aza) are being studied in clinical trials for treatment of diverse types of tumors (43). Furthermore, HDAC inhibitors alone can induce promoter de-methylation and de-repression (44). Because Kras-directed epigenetic silencing of Fas also requires HDACs (45), we asked whether DNA methylation inhibitors 5Aza or HDAC inhibitors such as Trichostatin A (TSA) or suberoylanilide hydroxamic acid (SAHA) can restore Fas expression in *Kras*$^{G12D/+}$ cells. All of these inhibitors significantly increased Fas mRNA expression in *Kras*$^{G12D/+}$ mouse lung cancer cells (Fig. 7*A*). Furthermore, two pan-HDAC inhibitors TSA and SAHA both significantly up-regulated Fas receptor level on the cell surface (Fig. 7*B*). Finally, we observed that TSA or SAHA pretreatment sensitized *Kras*$^{G12D/+}$ cells to Fas-mediated apoptosis in combination with Jo2 antibody (Fig. 7 *C* and *D*, and Fig. S7). These results suggest that exploiting the Fas activation as an Achilles' heel in oncogenic Kras cells using HDAC inhibitors can potentially provide a complementary approach to Kras inhibitors.

Discussion

Herein, we report CRISPR/Cas9-mediated genome editing of Kras in mouse and human lung cancer cells.

CRISPR Can Effectively Model Genetic Deletion of Oncogenes. Traditional technologies have limited ability to model complete silencing of oncogenes because of the low efficiency of homologous recombination without DNA breaks (18, 22). The CRISPR system is flexible and provides a rapid and facile genetic system to functionally investigate mechanisms of both *KRAS* and other "undruggable" oncogenes in lung cancer pathology. Using CRISPR to model genetic disruption of oncogenes will pave the road for identifying therapeutic targets (22, 23).

Cancer Cells Can Escape *KRAS* Oncogene Addiction and Survive Independent of KRAS. Cancer therapy can be improved by specifically modulating genes in resistance pathways. However, resistance to KRAS-targeted therapy is unknown due to the lack of effective small-molecule inhibitors for KRAS (16). Our data uncover KRAS independence upon complete KRAS knockout, which is consistent with our previous report that *Kras* mutant lung tumor can escape from RNAi-mediated *Kras* knockdown (16). Although *KRAS* knockout cells exhibited decreased colony formation ability, these cells are viable, implying mechanisms of resistance to KRAS inhibition or depletion. The recovery of tumors in the absence of Kras activity indicates potential resistance mechanisms to Ras inhibitors. Inhibiting KRAS alone, therefore, might be insufficient for treating KRAS-driven cancer in humans.

RNA-seq revealed a number of differentially expressed genes between oncogenic *Kras* and Kras knockout cells (Fig. 2). Understanding additional mechanisms of KRAS independence will be critical for tailoring treatment decisions in future generations of KRAS inhibitors being developed by the RAS initiative at the National Cancer Institute (3–5). Moreover, because we used *Kras*$^{G12D/+}$;*p53*$^{-/-}$ mouse lung cancer cells, it remains unclear how additional genetic lesions (e.g., *p53*$^{-/-}$) contribute to Kras independence. Future work is needed to investigate how Kras independence is affected by various genetic contexts.

Fas as an Achilles' Heel in Kras-Independent Cells. Because KRAS-independent cells are selectively sensitive to FAS-mediated apoptosis, understanding how KRAS regulates FAS might unveil treatment targets. Potential therapeutic options (such as siRNA) might exploit these targets to improve the efficacy of KRAS inhibitors. Moreover, a better understanding of the epigenetic regulation of *FAS* might allow us to turn *FAS* on and exploit it in other cancer types. Additional studies are also needed to determine whether Fas activation could mediate rejection of Ras-deficient tumor cells during early stages of lung tumor or organ development.

Tumor-Targeted Fas Activation Is Required in Vivo. Our results suggest that a Fas-activating antibody can selectively induce apoptosis in *Kras* knockout cells. However, normal somatic cells also express Fas receptor. For example, hepatocytes are highly sensitive to FAS-mediated apoptosis; the systemic injection of high-dose Jo2 antibody induces apoptosis in the liver (28). Thus,

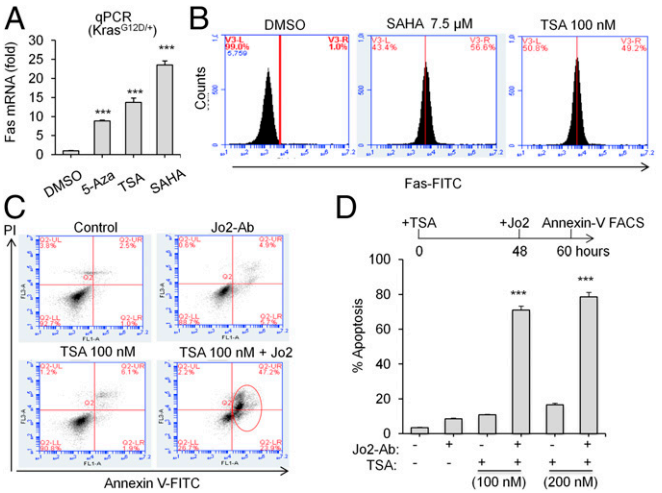

Fig. 7. A combination of HDAC inhibitor and Fas antibody induces apoptosis in oncogenic Kras cells. (*A*) qPCR showing reactivation of Fas in *Kras*$^{G12D/+}$ mouse KP lung cancer cells treated with 5-Aza (5 μM), TSA (10 nM), and SAHA (10 μM) for 48 h. DMSO control is set to 1. (*B*) Representative FACS histogram showing Fas expression on cell surface in the presence of TSA or SAHA. (*C*) FACS dot plot showing induction of apoptosis by Jo2 antibody in *Kras*$^{G12D/+}$ cells pretreated with TSA. (*D*) Quantitation of apoptosis measured by Annexin V/PI staining and FACS analysis. Error bars are SD (n = 3). ***P < 0.001.

FAS-activating antibody needs to be specifically delivered to tumor cells to avoid liver damage. Future studies will explore ways to tackle this challenge together with *KRAS* silencing. It may also be that endogenous FAS ligand excreted from tumor microenvironment can modulate apoptosis upon *KRAS* silencing in vivo.

In conclusion, our study has pinpointed *FAS* activation as a potential strategy to improve the efficacy of future KRAS inhibitors. Because KRAS mutations are prevalent in lung, colon, and pancreatic cancer and are associated with poor patient outcomes, these findings will be critical for developing effective ways to inhibit KRAS and prevent tumor relapse.

Materials and Methods

Vectors and Cloning. sgRNAs targeting mouse $Kras^{G12D}$ or human *KRAS* were designed using Broad Institute online tool (https://www.broadinstitute.org/rnai/public/analysis-tools/sgrna-design). The following sgRNA sequences were used: mouse $Kras^{G12D}$, 5′-GTGGTTGGAGCTGATGGCGT-3′, and human *KRAS*, 5′-GTAGTTGGAGCTGATGGCGT-3′. Oligos were annealed and cloned into the lenti.U6sgRNA.Cas9-2A-Puro vector using a standard BsmBI protocol. WZL-Hygro (Addgene; 18750) or WZL-HRAS-V12 (40) were gifts from Scott Lowe, Memorial Sloan Kettering Cancer Center, New York.

ChIP Assays. ChIP assays were performed as previously described (26) using DNMT1 (Abcam), EZH2 (CST) and H3K27me3 (Millipore), and H3K4Ac (Abcam) antibodies. ChIP products were analyzed by qRT-PCR using Fas promoter primer sets (Tables S1 and S2) corresponding to transcription start site (TSS) for EZH2, H3K27me3, and ∼1 kb upstream of TSS for DNMT1. Samples were normalized to input DNA, results were analyzed using the ΔΔCt method, and fold enrichment at target site was calculated with respect to IgG control.

Quantitative PCR. Total RNA was purified using an RNeasy Mini Kit (74104; Qiagen). Reverse transcription was performed and diluted cDNA was used as template for real-time PCR. TaqMan probes or SYBG primers were used to measure expression of mouse or human Fas (Tables S1 and S2).

Immunoblot Analysis. Cells were harvested and lysed in RIPA buffer with proteinase and phosphatase inhibitors. Cell lysate was quantified and equal amounts of protein were loaded into a 4–12% NuPage Bis-Tris gel (Life Technologies). The proteins isolated by the gel was then transferred to nitrocellulose membrane, blocked with blocking buffer (Odyssey), and then incubated with antibody against KRAS (sc-30; Santa Cruz) and Fas (Upstate; 05-351) overnight at 4 °C. An immunofluorescent secondary antibody (LICOR) was used for the Odyssey Imaging machine.

Statistical Analysis. All quantitation data were collected from at least three independent experiments, and the difference between groups were determined using two-tailed Student's *t* test, with $P < 0.05$ considered to be significant. All animal study protocols were approved by the University of Massachusetts Institutional Animal Care and Use Committee.

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