Supplemental Methods

Cell culture conditions
Cells were maintained in the following media: Dulbecco’s modified Eagle’s medium (Invitrogen, Carlsbad, CA) for HEK293T; RPMI-1640 medium (Invitrogen) for HT29 and LS513; Leibovitz L-15 Medium (Invitrogen) for SW403, SW480, and SW620; ATCC-formulated F-12K Medium for LoVo. All of the media were supplemented with 10% fetal bovine serum (FBS, Gibco-BRL, Grand Island, NY) and 1% ciprofloxacin hydrochloride (Sigma-Aldrich, St. Louis, MO) as antibiotic.

Transient transfection of plasmids encoding Cas9 and sgRNA
Cells were transfected with mixtures of the Cas9-puro and hU6-sgRNA plasmids at a weight ratio of 1:1 via Neon electroporation (Invitrogen), according to the manufacturer’s instructions. One day after transfection, puromycin (Gibco-BRL) was added to the culture media at a final concentration of 2.5 µg ml⁻¹ and cultures were incubated for two days to remove untransfected cells. Cells were analyzed three days after transfection.

Lentiviral vectors and lentivirus production
Lenti_inducible_sgRNA-GFP (Aubrey et al. 2015), which expresses sgRNA in a doxycycline-inducible manner, contains two bi-directional Bsmb1 sites for subsequent cloning of different sgRNA sequences. LentiCRISPR v2 (Addgene #52961; here referred to as Lenti_sgRNA-Cas9-Puro), lentiCas9-Blast (Addgene #52962; here referred to as Lenti_Cas9-Blast), lentiGuide-Puro (here referred to as Lenti_sgRNA-Cas9-Puro, Addgene #52963), and Lenti_inducible_sgRNA-GFP were used for lentivirus production as transfer plasmids (Supplemental Figure S1). HEK293T cells grown to 80~90% confluency on 100 mm dishes coated with 0.01% poly-L-lysine (Sigma-Aldrich) were transfected with a mixture (21 µg) of transfer plasmid, psPAX2 (Addgene; #12260), and pMD2.G (Addgene; #12259) at a weight ratio of 4:2:1 using Fugene HD (Roche, Mannheim, Germany) according to the manufacturer’s instructions. After 15 h of transfection, cells were refreshed with 12 mL of culture media. The supernatant, which contained virus, was collected at 39 (=15+24) and 63 (=15+48) hours after transfection, filtered through a 0.45-µm filter (Merck Millipore Ireland Tullagreen, Carigtwohill), and ultra-centrifuged for 1.5 h at 70,000 g (SW28 rotor of Beckman, ~120,000g) at 4°C to
concentrate the virus. The resulting pellet was then re-suspended in PBS. The lentiviral titers were determined using a Lenti-X™ qRT-PCR Titration Kit (Clontech).

**AAV vectors and AAV virus production**
To construct an AAV vector that expresses sgRNA, a PCR-amplified U6-sgRNA-scaffold cassette was inserted into the previously reported PX552 vector (Addgene #60958) digested with MluI and BstEII, which cut at sites between the two inverted terminal repeats. Modified PX552 (here referred to as AAV_sgRNA) and the miniCMV-Cas9-shortPolyA plasmid (here referred to as AAV_Cas9) obtained from Dr. Dirk Grimm (Heidelberg University Hospital, Germany) were used for AAV production (Supplemental Figure S1). AAV viruses were produced using previously described methods (Zolotukhin et al. 1999). Briefly, AAV_sgRNA or AAV_Cas9 was cotransfected into HEK293T cells together with pAAV-RC2 (AAV serotype2, rep and cap, Cell Biolabs, VPK-402) and helper DNA (Cell Biolabs, VPK-402) at a 1:1:1 molar ratio using the CaPO4 transfection method (Zolotukhin et al. 1999). At 48 h post-transfection, cells were harvested by centrifugation at 1,140g for 10 min. Cells were then lysed in a solution of 0.15 mM NaCl, 50 mM Tris-HCl (pH 8.5) by three freeze/thaw cycles. Benzonase (Invitrogen) was added to the mixture (50 U/ml, final concentration) and the lysate was incubated for 30 min at 37°C. The lysate was clarified by centrifugation at 3,700 g for 20 min, and the virus-containing supernatant was considered the crude lysate. The infectious AAV particles were purified by ultracentrifugation through an iodixanol density gradient in a Type 70 Ti rotor (Beckman-Coulter Instruments, Palo Alto, CA) at 350,000g for 1 h at 18°C. The AAV virus titer was determined by SYBR Green I-based quantitative PCR (Sigma-Aldrich).

**In vitro lentiviral transduction**
For viral infection, cells were seeded into six-well plates at 1 x 10⁵ cells/well in the presence of 8 μg/ml polybrene and incubated with virus-containing medium. This medium was replaced with fresh culture medium 24 hours after infection. Two days after transduction of lentivirus expressing Cas9, the cultures were supplemented with 10 μg/ml blasticidin (invivoGen, San Diego, CA) and incubated for 7 days to remove untransduced cells. The Cas9-expressing cells were transferred to new 6-well plates and transduced with lentivirus expressing sgRNA. Untransduced cells were removed by adding 2 μg/ml puromycin (Gibco-BRL) to the culture medium 24 h after infection and incubating for 48 h.
Subsequently, the double-transduced cells were counted and subjected to a focus-forming assay and an anchorage independence culture assay.

**Animal experiments**

All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) at Yonsei University College of Medicine (Seoul, Korea). All mice were maintained in the experimental animal facility under specific pathogen-free conditions and provided with sterilized food (Certified Diet MF; Oriental Yeast, Osaka, Japan) and filtered tap water ad libitum.

**Doxycycline-induced expression of 35T9P17 sgRNA in vivo**

Two million cancer cells double-transduced with both lentivirus expressing Cas9 and lentivirus expressing 35T9P17 sgRNA in a doxycycline-inducible manner were subcutaneously injected into the flanks of 5-week-old athymic male BALB/c nude mice (six mice per group) and were allowed to form tumors over two weeks. Doxycycline was added to the drinking water, which was supplemented with 1% sucrose, at 2 mg/ml for in vivo induction of sgRNA expression. Tumor sizes were measured every two or three days using a caliper (Mitutoyo). The tumor volume (V) was calculated using the formula (S × S × L) × 0.5, where S = short - and L = long dimensions (Britten et al. 1999). Mice were sacrificed 5 weeks after the tumor cell injection, and the tumors were surgically isolated and weighed.

**Supplemental References**

Aubrey BJ, Kelly GL, Kueh AJ, Brennan MS, O’Connor L, Milla L, Wilcox S, Tai L, Strasser A, Herold MJ. 2015. An inducible lentiviral guide RNA platform enables the identification of tumor-essential genes and tumor-promoting mutations in vivo. *Cell Rep* **10**: 1422-1432.

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Zolotukhin S, Byrne BJ, Mason E, Zolotukhin I, Potter M, Chesnut K, Summerford C, Samulski RJ, Muzyczka N. 1999. Recombinant adeno-associated virus purification using novel methods improves infectious titer and yield. *Gene Ther* **6**: 973-985.
Supplemental Figures

Supplemental Figure S1. KRAS mutations, guide RNA nomenclature, and vector maps. (A) KRAS mutations, for which sgRNAs were designed and tested in this study, are shown. These KRAS mutations are strongly associated with cancer development. The black line represents KRAS genomic DNA and E2 indicates exon 2. (B) SgRNA nomenclature. The boxes represent, in order, the following details: (i) a mutated nucleotide and its locus (e.g., if the mutation is c.35G>T, then it is referred to as 35T), (ii) the distance from the PAM to the mutation locus, (iii) the location of PAM relative to the mutation locus (if the mutation locus is located to the left of PAM, it is referred to as plus (P), or to the right, minus (M)), and (iv) the total length of the sgRNA (here, 17 bases).
Supplemental Figure S2. Structure and working mechanism of NHEJ reporters.

mRFP is constitutively expressed by the CMV promoter ($P_{CMV}$), whereas eGFP is not expressed without CRISPR/Cas9 activity due to its out-of-frame sequence and the existence of stop codon before eGFP. When a double-stranded break is introduced into the target sequence by CRISPR/Cas9, this break can be repaired by error-prone nonhomologous end joining, which often leads to indel generation. The indels can result in frame-shifts of the two eGFP genes, potentially leading to the expression of one or the other.
Supplemental Figure S3. Reporter-based selection of sgRNAs that target mutant KRAS. The ratios of eGFP+ mRFP+/eGFP+ cells after transfection of reporters with mutant (c.34G>T (A), c.34G>C (B), or c.35G>C (C)) or wild-type target sequences, which
reflect guide RNA activities at the target sequences, are shown on the left in the two-dimensional graphs. Black and red lines in the graphs represent ratios of eGFP+mRFP+/eGFP+ cells for mutant vs. wild-type KRAS target sequences with values of 1 and 3, respectively. The target sequence of each sgRNA is shown on the right side; the KRAS point mutation site is shown in red and the protospacer adjacent motif sequence is in blue. SgRNAs selected for subsequent studies are indicated with black arrows in the two-dimensional graphs and their names are shown in bold.
Supplemental Figure S4. Viral vector maps. (A-D) Maps of lentiviral vectors that express sgRNA (A, B) in a constitutive (A) or inducible (B) manner, of a lentiviral vector that expresses Cas9 (C), and of a lentiviral vector that expresses both Cas9 and sgRNA (D). (E, F) Maps of adeno-associated viral (AAV) vectors that express sgRNA (E) and Cas9 (F). Psi, packaging signal; RRE, rev response element; WPRE, posttranscriptional regulatory element of woodchuck hepatitis virus; cPPT, central polypurine tract; U6 pol III promoter; gRNA, guide RNA; EF1α, elongation factor 1a promoter; PuroR, puromycin resistance gene; H1, H1 promoter; TetO, tetracyclin operator; Ub, ubiquitin promoter; TetR, tetracyclin repressor; T2A, T2A peptide; EGFP, Enhanced Green Fluorescent Protein; CMV, the promoters for cytomegalovirus; BlastR, blasticidin resistance gene.
Supplemental Figure S5. Cas9 and sgRNA-directed selective and efficient disruption of mutant KRAS in cancer cells. Cas9 and sgRNAs were delivered into cancer cells and the indel frequencies at the endogenous target sequences were evaluated by deep sequencing and plotted on a log scale. Untreated cells were used as the control. The names of the cancer cell line and their related mutations are shown at the top of each graph. (A-F) Indel frequencies in cancer cells after delivery of Cas9 and sgRNA. Error bars represent SEM. Hetero., heterozygous; Homo., homozygous.
Supplemental Figure S6. The sequence frequencies of wild-type *KRAS*, mutant *KRAS*, and indels (individual experimental data). Cas9 and sgRNAs were delivered into cancer cells and the indel frequencies at the endogenous target sequences were evaluated by deep sequencing. Untreated cells were used as the control. Each horizontal bar represents an independent experiment. The names of the cancer cell lines and their related mutations are shown at the top of each graph. Hetero., heterozygous; Homo., homozygous. Red = Indels, Green = Wild-type *KRAS* (unedited), Purple = Mutant *KRAS* (unedited).
Supplemental Figure S7. Activity of the 35T9P17 sgRNA expressed in a doxycycline-inducible manner in cancer cells. Cells were transduced with one lentiviral vector that expresses Cas9 and another lentiviral vector that expresses sgRNAs in doxycycline-inducible manner. Cells were analyzed after being cultured in the presence or absence of doxycycline for 72 hrs. Indel frequencies determined by deep sequencing are shown in scattered-dot graphs with a linear (A) or log (B) scale. Hetero., heterozygous; Homo., homozygous.
Supplemental Figure S8. Mutation patterns of indels generated in mutant KRAS. (A) DNA sequences from the wild-type and mutant clones of SW403 (heterozygous c.35G>T; cell line edited by 35T9P17), with CRISPR/Cas9 recognition sites shown in red and the protospacer adjacent motif (PAM) sequence shown in bold blue characters. Hyphens and lower-case letters indicate deleted and inserted bases, respectively; numbers in the first set of parentheses indicate the frequency of each clone; the number of inserted or deleted bases are indicated with plus or minus symbols in the second parentheses. The proportions of out-of-frame and in-frame mutations are indicated in the pie graphs in orange (out-of-) and dark red (in-frame) colors. Mutation frequencies were obtained by dividing the number of mutant clones by the total number of clones. Hetero., heterozygous; Homo., homozygous. Equivalent descriptions apply to (B) SW480 (homozygous c.35G>T; cell line edited by 35T9P17), (C) SW620 (homozygous c.35G>T; cell line edited by 35T9P17), (D) LS513 (heterozygous c.35G>A; cell line edited by 35A9P17), and (E) LoVo (heterozygous c.38G>A; cell line edited by 38A9P17).
Supplemental Figure S9. Targeting mutant KRAS with CRISPR-Cas9 inhibits cancer cell survival, proliferation, and tumorigenicity in vitro. Cancer cells containing KRAS mutations were subjected to colony forming (A-B), soft agar (C-D), and MTS (E-F) assays after lentiviral delivery of Cas9 and sgRNAs targeting mutant KRAS. A completely different sequence-targeting guide RNA without activity was used as the control (negative control). Hetero., heterozygous; Homo., homozygous. Error bars represent SEM. *p < 0.05, **p < 0.01, ***p < 0.001. (A-B) Colony forming assay. Representative images of wells after 2% crystal violet staining are shown at the top of each graph. (C-D) Soft agar assay. Representative images of formed colonies are shown at the top of each graph. Scale bar = 100 μm. (E-F) MTS assay. One day after the final transduction, untransduced cells were removed using puromycin selection for 24 hr, after which 5,000 cells per sample were plated onto 96 well plates. Cell proliferation was determined by use of MTS reagents 48 hrs after plating. The relative number of cells in cultures transduced with active versus
negative control sgRNAs was determined by normalizing the optical density at 490 nm of each MTS reaction to the average optical density of the negative control reactions.
Supplemental Figure S10. Cancer cells containing KRAS mutations were subjected to the colony forming assay after lentiviral delivery of Cas9 and sgRNAs targeting mutant KRAS. Cancer cells were transduced with a lentiviral vector expressing the 35T9P17 sgRNA in a doxycycline-inducible manner and a lentiviral vector that constitutively expresses Cas9. Cells were analyzed after culturing in the presence or absence of doxycycline for 72 hrs. Hetero., heterozygous; Homo., homozygous. Error bars represent SEM. *p < 0.05, **p < 0.01, ***p < 0.001.
Supplemental Figure S11. RTCA (real-time cell analyzing) cell index curves of mutant KRAS cancer cells after expression of Cas9 and mutant KRAS-targeting sgRNA. RTCA was performed on KRAS mutant cancer cells (SW403 (A), SW480 (B), and SW620 (C)) and KRAS wild-type cells (HT29) after lentiviral delivery of Cas9 and the 35T9P17 sgRNA (a-c) or the 35T9P17, 35A9P17, or 38A9P17 sgRNAs (D). One day after the final transduction, untransduced cells were removed by puromycin selection for 24 hr, after which 5,000 cells per sample were plated onto E-plates (16 wells) and monitored for 72 hours. Cells transduced with a completely different sequence-targeting guide RNA without activity, as well as an untreated population, were used as controls (negative control and untreated). Hetero., heterozygous; Homo., homozygous.
**Supplemental Figure S12. Targeting mutant KRAS with CRISPR-Cas9 blocks tumor growth in vivo.** SW480 cancer cells were transduced with a lentiviral vector expressing the 35T9P17 sgRNA in a doxycycline-inducible manner and a lentiviral vector that constitutively expresses Cas9. Transduced cells were subcutaneously injected into immunodeficient mice to allow tumor formation over 14 days, after which mice were treated with doxycycline to induce expression of 35T9P17. (A) Representative photographs of the mice after 12 days of doxycycline treatment (26 days after tumor cell injection). (B, C) Representative photographs (B) and weights (C) of tumors removed from euthanized mice after 12 days of doxycycline treatment. Error bars represent SEM. Red scale bar = 1 cm. *p < 0.05, **p < 0.01, ***p < 0.001. Homo., homozygous.
Supplemental Figure S13. Representative photographs of athymic mice with tumors after intratumoral injections of lentivirus expressing Cas9 and sgRNA. Cancer cells containing mutant KRAS (A) or wild-type KRAS (B) were subcutaneously injected into immunodeficient mice, after which tumors were allowed to form over 14 days. Lentivirus expressing both Cas9 and sgRNA (35T9P17) (Lenti_Cas9-sgRNA) or control lentivirus expressing only Cas9 (Lenti_Cas9) were injected into the tumors three times every 3 days. Representative photographs of athymic mice with tumors 12 days after the first lentiviral injection are shown.
Supplemental Figure S14. Intratumoral delivery efficiency of lentivirus in vivo. SW403 cancer cells containing mutant KRAS were subcutaneously injected into immunodeficient mice, after which tumors were allowed to form over 14 days. Lentivirus expressing both Cas9 and sgRNA (35T9P17) (Lenti_Cas9-sgRNA) or negative control lentivirus expressing only Cas9 (Lenti_Cas9 only) were injected into the tumors three
times every 3 days. Tumor tissue was harvested 6 days after the first lentiviral injection and subjected to various analyses. Immunohistochemical staining was performed using an antibody against puromycin acetyltransferase (PuroR), a protein that is expressed by the lentiviral vector for sgRNA and Cas9 expression. (A-D) Representative pictures of microscopic evaluation of immunohistochemical staining against PuroR (A, B) and H and E staining (C, D). Scale bar = 100 μm. (E) The percentages of PuroR-positive tumor cells relative to the total tumor cells are shown. The numbers of PuroR-positive and negative tumor cells were quantified from >3 regions per tumor. (F) The indel frequencies at KRAS target sites in tumor cells, determined by deep sequencing, are shown.
Supplemental Figure S15. Adeno-associated virus-mediated intratumoral delivery of Cas9 and sgRNA targeting mutant KRAS suppresses tumor growth in vivo. SW403 cancer cells containing mutant KRAS (c.35G>T) were subcutaneously injected into immunodeficient mice, after which tumors were allowed to form over 14 days. Adeno-associated virus (AAV) expressing both Cas9 and sgRNA (35T9P17) (AAV_Cas9-sgRNA) or control AAV expressing GFP (AAV_GFP) were injected into the tumors three times every 3 days. (A) Tumor growth curves. Black arrowheads indicate the times when the lentivirus was injected. The number of tested tumors \( n = 4 \). (B) Representative photographs of athymic mice with tumors 14 days after the first AAV injection. (C, D) Representative photographs (C) and weights (D) of tumors removed from euthanized mice 12 days after the first lentiviral injection. Red scale bar = 1 cm. Error bars represent SEM. \( **p < 0.01 \).
Supplemental Figure S16. Secondary mutation frequencies in human cells after lentiviral delivery of Cas9 and KRAS-targeting sgRNA. Cas9 and KRAS-targeting sgRNAs (35T9P17) were lentivirally delivered into cultured cells (A, B, D, E) or tumors in mice composed of human cancer cells containing mutant KRAS (SW403) or wild-type KRAS (HT29) (C, F). Secondary point mutations at the endogenous target sequences were evaluated by deep sequencing. Among the secondary point mutations, frequencies of gain-of-function mutations (A-C) and those of nonsynonymous mutations that were not included in a previously reported list of gain-of-function mutations (uncharacterized, D-F) are shown. The list of reported gain-of-function mutations that were considered consists of G12D, G12V, G12C, G13D, G12R, G12A, and G12S. Cells transduced with lentivirus that expresses only Cas9 (Lenti-Cas9 only) were used as the control. The names of the cancer cell lines and their related mutations are shown at the top of each graph. 15 d, 15 days after the first lentiviral injection; 6 d, 6 days after the first lentiviral injection; Hetero., heterozygous; Homo., homozygous.