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Supplemental Information

DIRAS3 (ARHI) Blocks RAS/MAPK Signaling

by Binding Directly to RAS

and Disrupting RAS Clusters

Margie N. Sutton, Zhen Lu, Yao-Cheng Li, Yong Zhou, Tao Huang, Albert S. Reger, Amy M. Hurwitz, Timothy Palzkill, Craig Logsdon, Xiaowen Liang, Joe W. Gray, Xiaolin Nan, John Hancock, Geoffrey M. Wahl, and Robert C. Bast Jr.

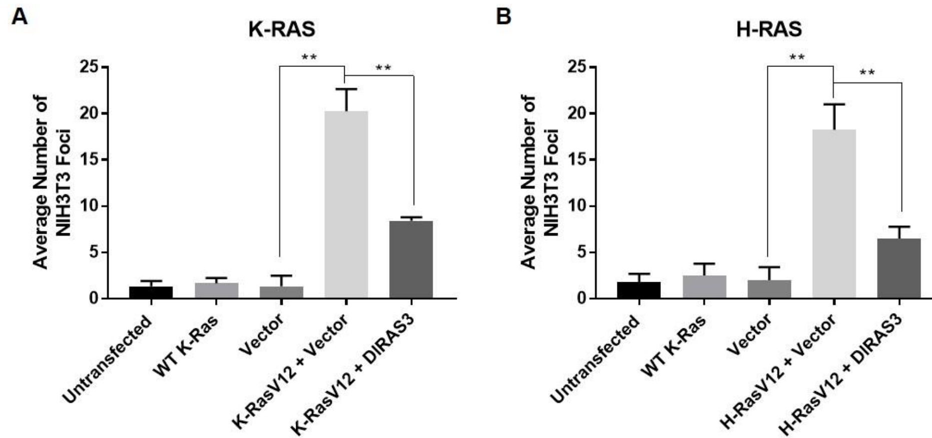


Figure S1. Focus Formation of NIH3T3 cells transformed with oncogenic K-RAS and H-RAS. Related to Figure 1. A-B. DIRAS3 inhibits K-RAS and H-RAS induced transformation of murine NIH3T3 cells compared to untransfected, WT-RAS and vector only transfected cells. Cells were plated in 60 mm dishes and transfected with 10 µg of each DNA plasmid for 24 hours prior to being separated into dishes for focus formation and clonogenic selection by G418. Transformed foci were counted at 10x magnification as they appeared within two 10x10 mm areas per plate. The columns indicate the mean, and the error bars indicate the S.D. (**$p<0.01$ *$p<0.05$). Clonogenic selection by G418 was used to ensure equal transfection efficiency.

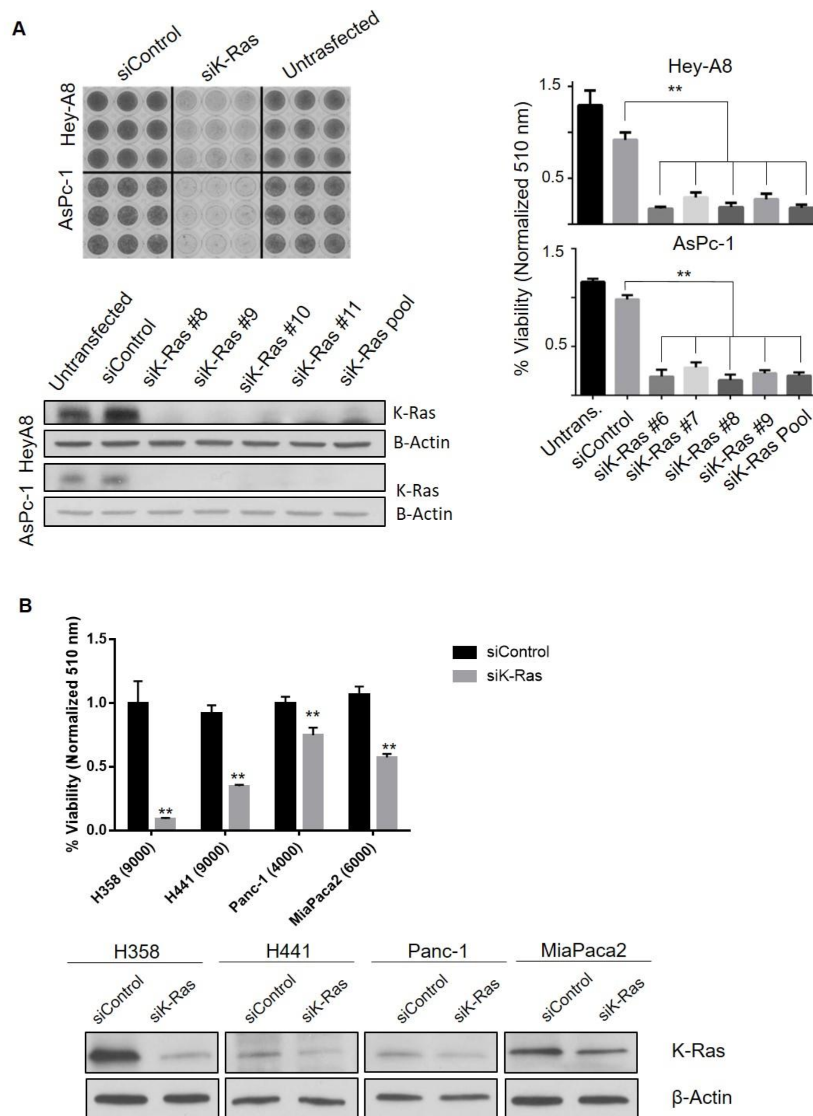
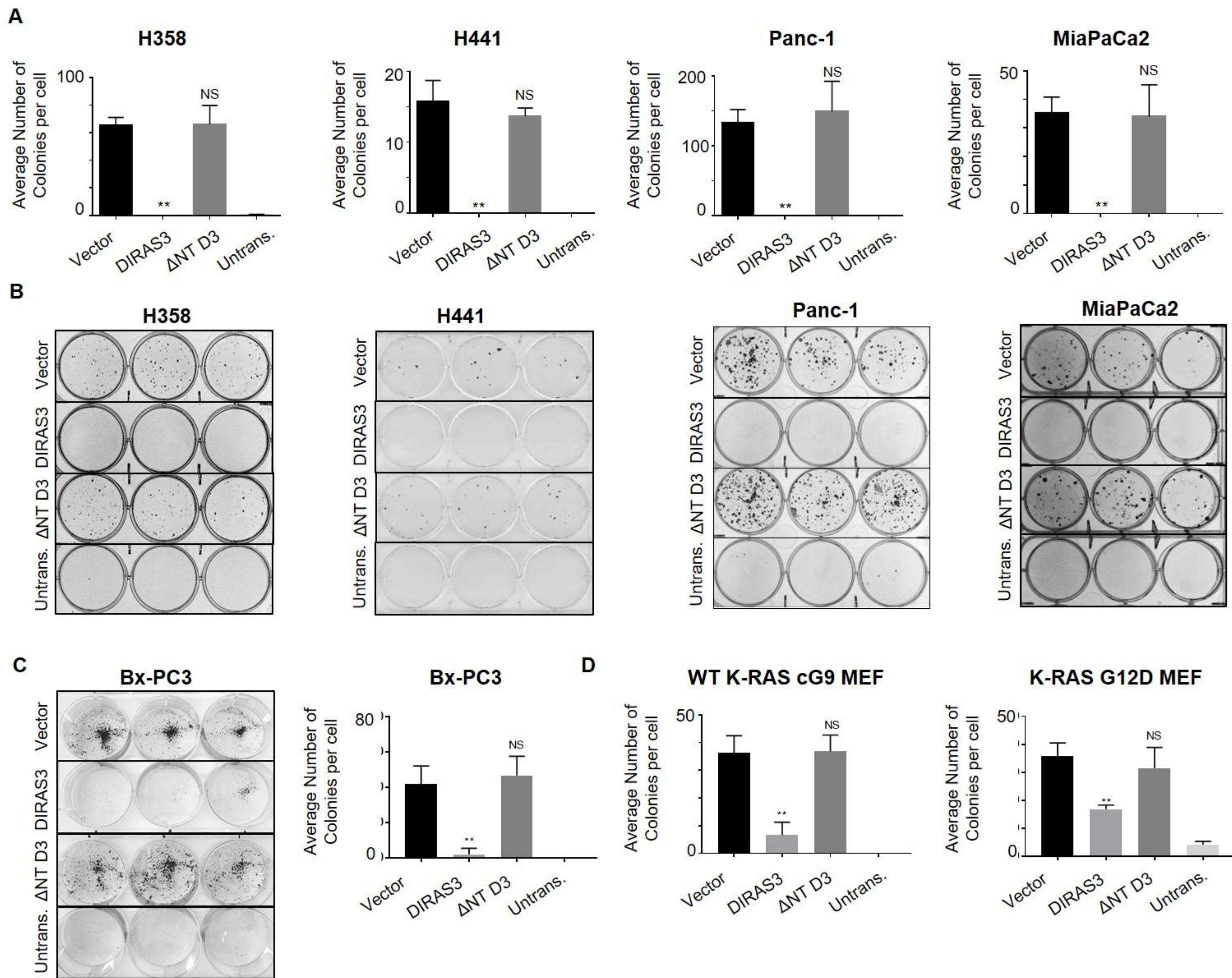


Figure S2. Knockdown of K-RAS by siRNA inhibits growth of several K-RAS mutant cancer cell lines. Related to Figure 2. A. Reverse transfection (2,000 cells per well) of 4 different K-RAS siRNAs or a pooled mixture was a performed in a 96-well plate. Cells were then incubated for 72 hours before fixation and staining by sulforhodamine B for cell viability. Data were obtained from three independent experiments. K-RAS protein levels were measured by western blot analysis. The columns indicate the mean, and the bars indicate the S.D. (**$p<0.01$). SRB assays were performed following knockdown of K-RAS in several additional cell lines and their

corresponding knockdown efficiency, as determined by western blot analysis. **B.** Reverse transfection (2,000 cells per well) of a pooled mixture of K-RAS siRNA was a performed in a 96-well plate. Cells were then incubated for 72 hours before fixation and staining by sulforhodamine B for cell viability. Data were obtained from three independent experiments. K-RAS protein levels were measured by western blot analysis to determine the knockdown efficiency. The columns indicate the mean, and the bars indicate the S.D. (**$p<0.01$).

Figure S3



SFigure S3. Transient re-expression of DIRAS3, but not ΔNT DIRAS3, inhibits RAS-driven growth in several K-RAS and B-RAF mutant cancer cell lines and RASless MEFs with exogenous expression of wt K-RAS or oncogenic K-RAS. Related to Figure 2. A-B. H358, H441, Panc-1 and MiaPaca2 cells were transiently transfected with an empty vector, DIRAS3, or ΔNT DIRAS3 DNA plasmid for 24 hours prior to re-seeding into a 6-well plate, in triplicate. Selection of transfected clones was performed with G418, using an untransfected well as a control. Two weeks following selection, colonies were stained with Coomassie blue (B) and counted (A).

The columns indicate the mean and the bars indicate the S.D. (**$p<0.01$). **C.** Bx-PC3 cells were transiently transfected with an empty vector, DIRAS3, or ΔNT DIRAS3 DNA plasmids for 24 hours prior to re-seeding in triplicate into a 6-well plate. Selection of transfected clones was performed with G418, using an untransfected well as a control. Two weeks following selection, colonies were stained with Coomassie blue (left) and counted (right). The columns indicate the mean and the bars indicate the S.D. (**$p<0.01$). **D.** Isogenic RAS-dependent cell lines re-expressing WT or oncogenic K-RAS 4B G12D were transiently transfected with an empty vector, DIRAS3, or ΔNT DIRAS3 DNA plasmid for 24 hours prior to re-seeding into a 6-well plate, in triplicate. Selection of transfected clones was performed with G418, using an untransfected well as a control. Two weeks following selection, colonies were stained with Coomassie blue and counted. The columns indicate the mean and the bars indicate the S.D. (**$p<0.01$).

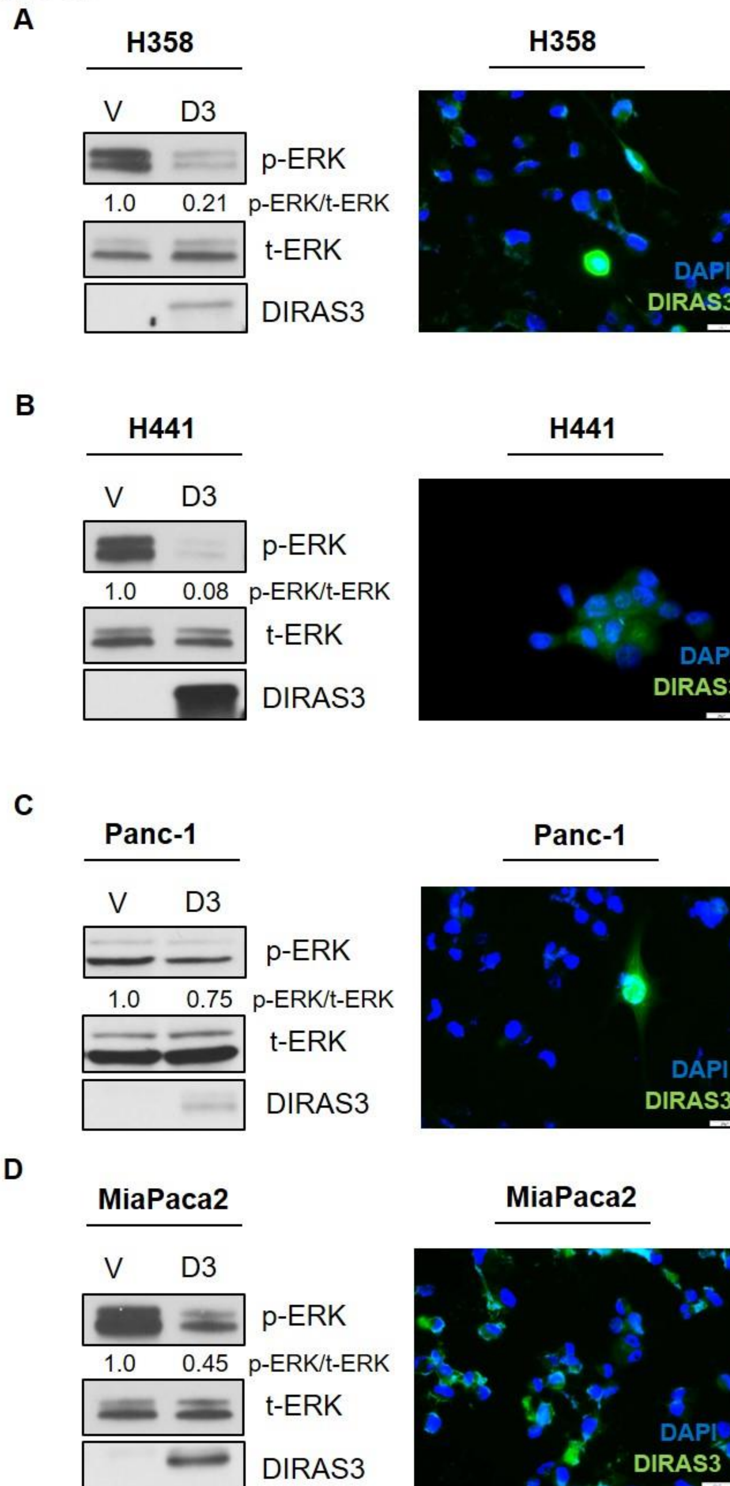


Figure S4. Transient re-expression of DIRAS3 inhibits RAS/MAPK signaling. Related to Figure 2. A-D. H358 (A), H441 (B), Panc-1 (C) and MiaPaca2 (D) cells were transiently

transfected with an empty vector or DIRAS3 DNA plasmid for 24 hours and cell lysate was collected for western blot analysis and probed as indicated. Densitometry was measured with ImageJ and experiments were performed at least two times. The ratio of p-ERK to t-ERK was calculated and normalized to the vector control. Immunofluorescence staining was performed to document transfection efficiency. Anti-GFP signal represents DIRAS3, DAPI represents nuclei. Scale bars: 20μm.

Figure S5

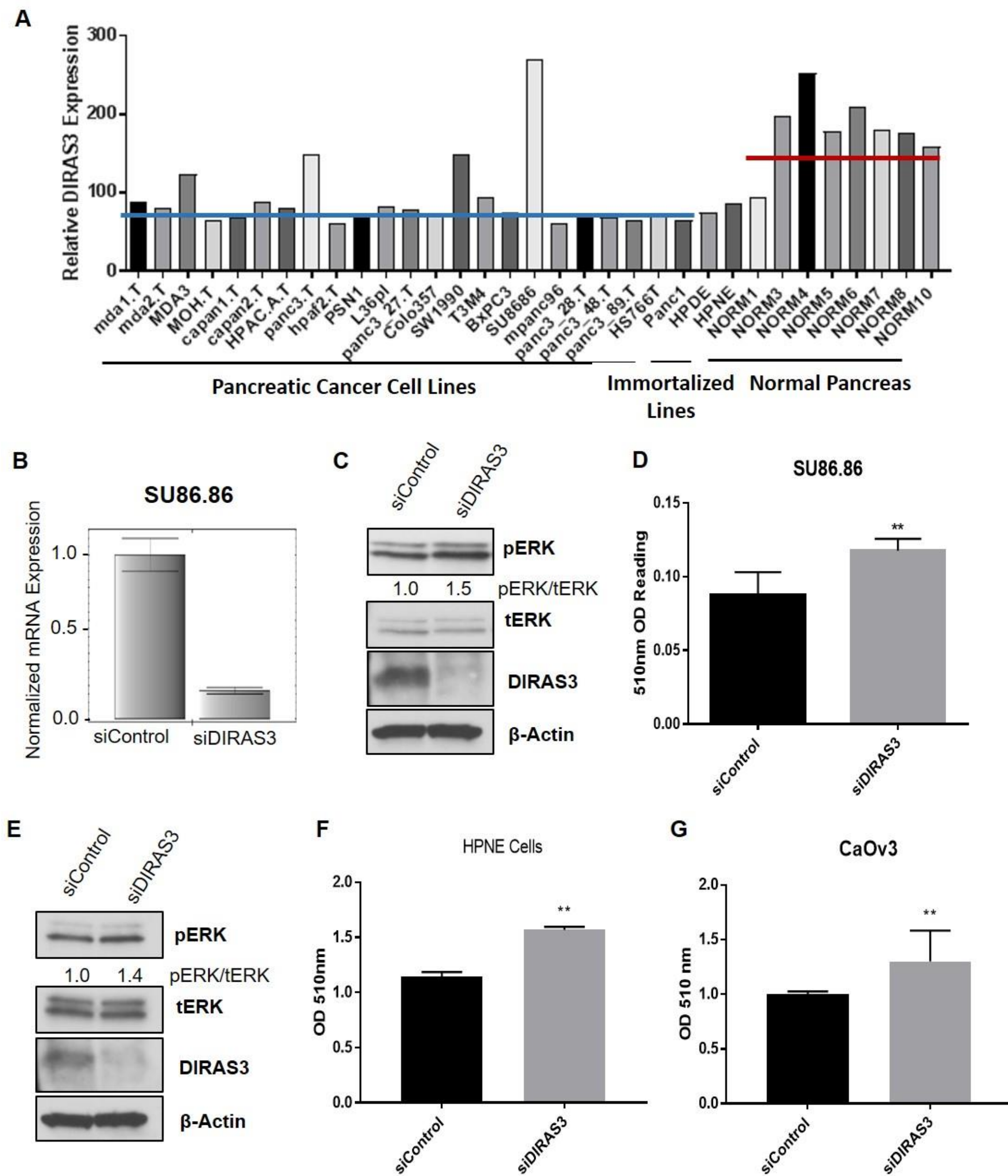


Figure S5. Transient knockdown of DIRAS3 increases cell viability and p-ERK signaling of SU86.86 pancreatic cancer cells and HPNE normal cells. Related to Figure 2. A. Relative DIRAS3 mRNA expression was determined by affymetrix array of several pancreatic cancer cell lines as compared to normal pancreas or immortalized lines. **B.** qPCR analysis of DIRAS3 mRNA

of SU86.86 cell line following siRNA knockdown of DIRAS3 at 72 hours. **C.** Reverse transfection of SU86.86 pancreatic cancer cells with pooled DIRAS3 siRNA was performed for 72 hours prior to harvesting the cells for western blot analysis. Blots were probed as indicated. Densitometry was measured with ImageJ and experiments were performed at least three times. The amount of p-ERK was calculated and normalized to the t-ERK. To visualize knockdown efficiency, 80 μg of protein lysate was used and probed with anti-DIRAS3 antibody. **D.** Reverse transfection (2,000 SU86.86 cells per well) of a pooled mixture of DIRAS3 siRNA was a performed in a 96-well plate. Cells were then incubated for 72 hours before fixation and staining by sulforhodamine B for cell mass. Data were obtained from three independent experiments. The columns indicate the mean, and the bars indicate the S.D. (**p<0.01). **E.** Reverse transfection of HPNE pancreatic cancer cells with pooled DIRAS3 siRNA was performed for 72 hours prior to harvesting the cells for western blot analysis. Blots were probed as indicated. Densitometry was measured with ImageJ and experiments were performed at least three times. The amount of p-ERK was calculated and normalized to the t-ERK. **F-G.** Reverse transfection (2,000 HPNE cells per well **(F)** and 5,000 CaOv3 cells per well **(G)**) of a pooled mixture of DIRAS3 siRNA was a performed in a 96-well plate. Cells were then incubated for 72 hours before fixation and staining by sulforhodamine B for cell viability. Data were obtained from three independent experiments. The columns indicate the mean, and the bars indicate the S.D. (**p<0.01).

Figure S6

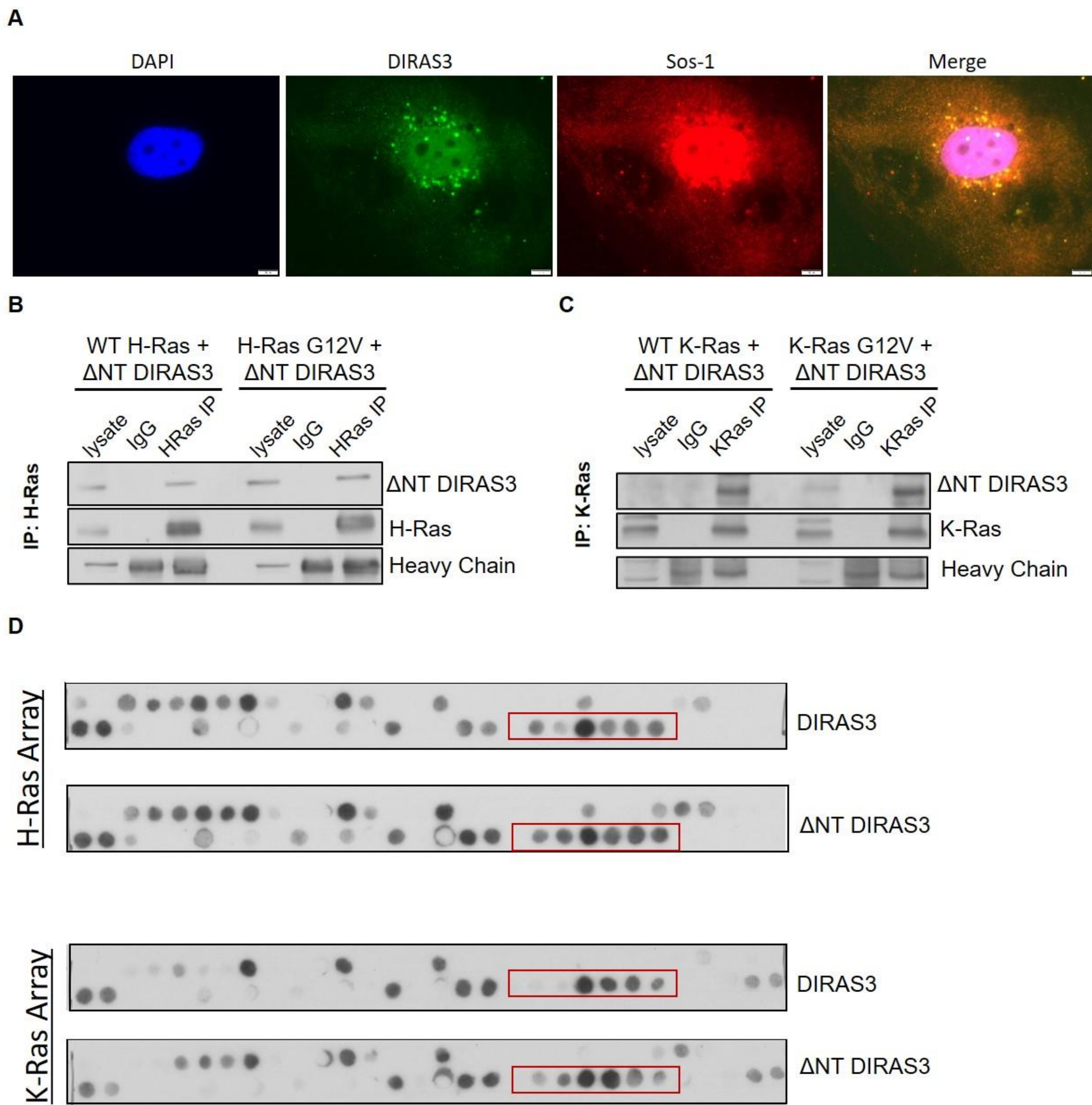


Figure S6. ΔNT DIRAS3 co-immunoprecipitates with mutant and wild type H-RAS and K-RAS. Related to Figures 3 and 4. A. DIRAS3 co-localizes with Sos-1 by immunofluorescence. U2OS-701 cells were treated with DOX for 24 hrs. Immunofluorescence staining of expressed DIRAS3 and endogenous Sos-1 was analyzed by confocal microscopy. Scale bars: 30 μM. **B-C.**NIH3T3 cells were transiently transfected with wt RAS or RASG12V (H-RAS and K-RAS) and

ΔNT DIRAS3 DNA plasmids for 24 hours before lysate was harvested. 1.0 mg of protein lysate was used to immunoprecipitate H-RAS **(B)** and K-RAS **(C)** and western blot analysis was performed. Blots were probed for ΔNT DIRAS3 (~21 kDa) and then stripped and probed for H-RAS. **D.** 15-mer peptide arrays generated for H-RAS and K-RAS were probed with DIRAS3 and NTD recombinant protein.

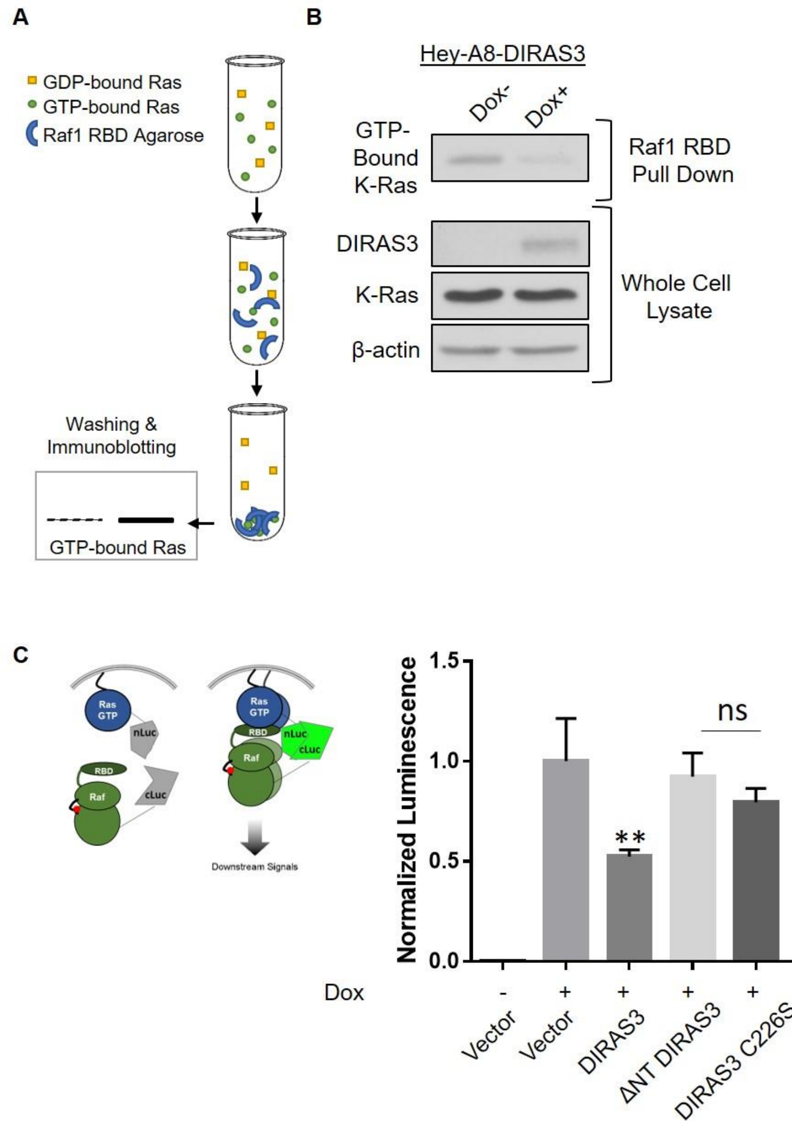


Figure S7. DIRAS3 inhibits GTP-bound RAS and subsequent RAS:Raf interaction. Related to Figures 5 and 6. A. Assay schematic for Raf1 RBD agarose pull-down assay to determine levels of GTP-bound RAS. **B.** Hey-A8-DIRAS3 cells were plated at 70% confluency in a 100 mm dish and treated with our without doxycycline to induce DIRAS3 expression for 24 hrs. Cells were lysed and GTP-bound RAS was determined by Raf1 RBD (RAS binding domain) pull down. Immunoblotting was performed and probed as indicated. **C.** ReBiL cells were plated at 0.3 x 10^6

cells/well in a 6-well plate and transfected with 3 μg of Vector, DIRAS3, ΔNT DIRAS3 (NTD) or DIRAS3 C226S plasmid DNA. 24 hours post transfection, cells were re-seeded into 96-well plates at a density of 5,000 cells/well. Doxycycline was added to induce the split luciferase constructs and 24 hours later the luminescent signals were determined for K-RASG12D:BRaf interaction. Data was obtained from three independent experiments performed in triplicate. Columns indicate the mean, and the bars indicate the S.D. (**$p<0.01$).

Figure S8

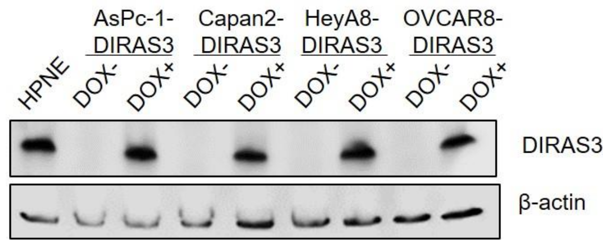
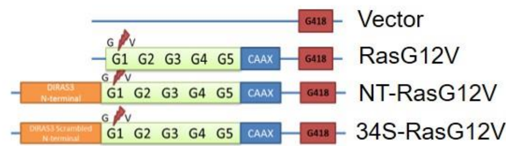


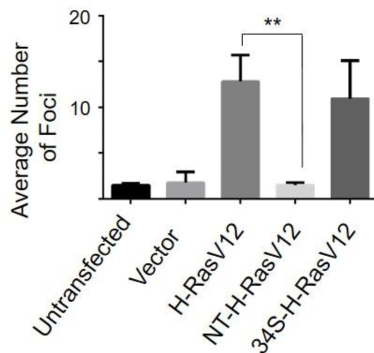
Figure S8. DIRAS3-inducible cell lines re-express DIRAS3 protein at similar levels to endogenous HPNE cells. Related to Figures 2-6. Western blot analysis of HPNE or DIRAS3-inducible cells at 24 hours post induction of DIRAS3 with doxycycline (500 ng/mL).

Figure S9

A



B



C

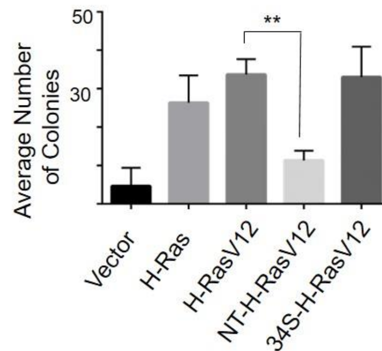


Figure S9. DIRAS3 N-terminus can reverse H-RAS driven transformation and anchorage independent growth. Related to Figures 1 and Figure 6. A. Cartoon of constructs in pcDNA3.1 Vector that were used for the following experiments. The 34-amino acid N-terminus of DIRAS3 (NT-H-RASV12) or a scrambled 34-amino acid control (34S-H-RASV12) were fused directly to the N-terminus of mutant H-RAS. **B.** DIRAS3 N-terminus inhibits H-RAS induced transformation

of NIH3T3 cells. Cells were plated in 60 mm dishes and transfected with 10 μg of each DNA plasmid for 24 hours prior to being separated into dishes for foci formation and clonogenic selection by G418. Transformed foci were counted at 10x magnification as they appeared within two 10x10 mm areas per plate. The columns indicate the mean, and the error bars indicate the S.D. (**$p<0.01$ *$p<0.05$). Clonogenic selection by G418 was used to ensure equal transfection efficiency. **C.** DIRAS3 N-terminus inhibits anchorage independent growth of MCF10a breast epithelial cells transformed with H-RAS. Cells were plated in 6-well plates and transfected with 3 μg of each DNA plasmid for 24 hours prior to being re-seeded at a cell density of 1.0×10^5 in soft agar. Cells were grown for two weeks and colonies were quantified. The assay was performed three times with technical triplicates for each experiment. The columns indicate the mean, and the error bars indicate the S.D. (**$p<0.01$).