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Supplemental Information

Concurrent Mutations in *STK11* and *KEAP1* Promote Ferroptosis Protection and SCD1 Dependence in Lung Cancer

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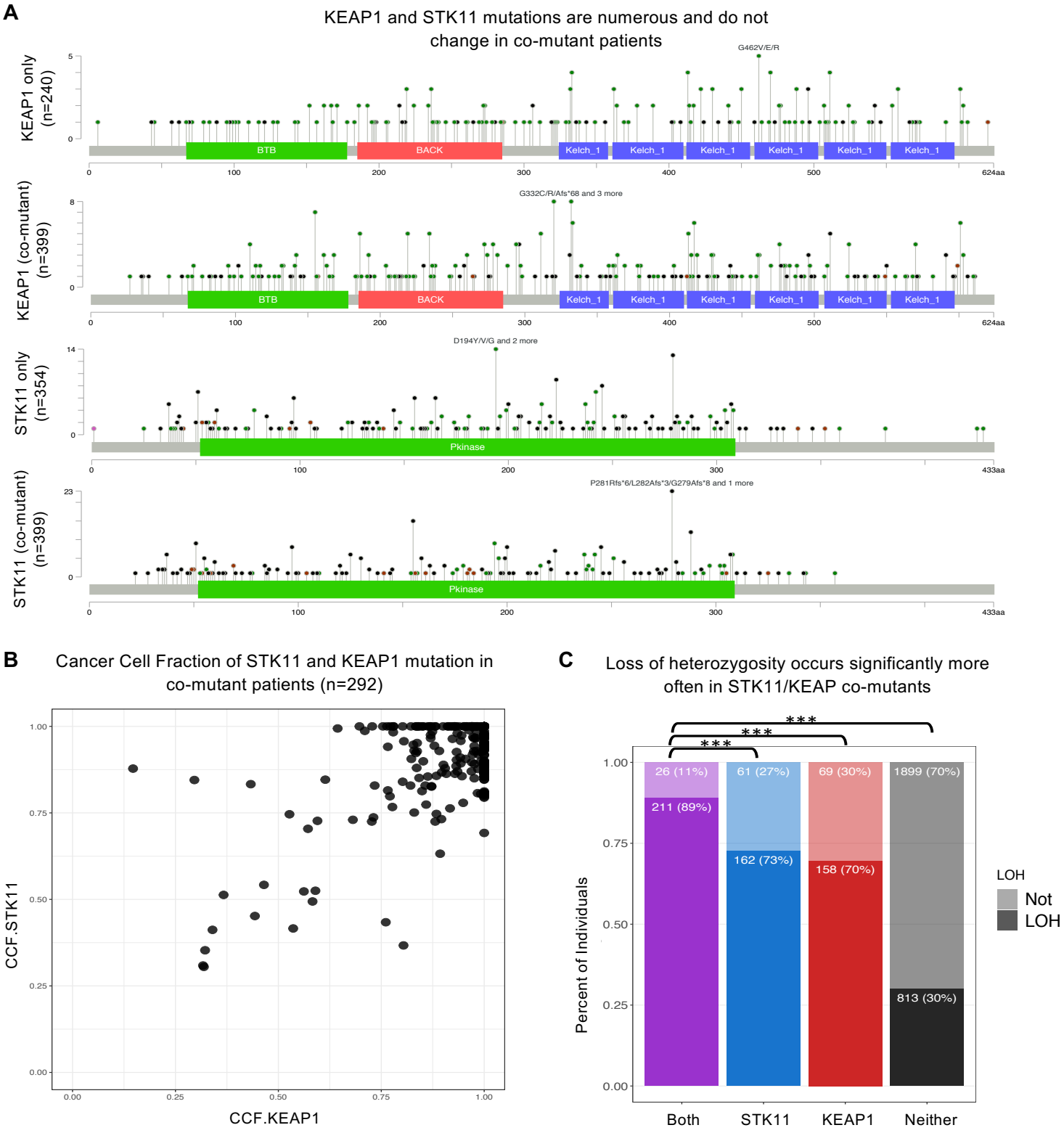
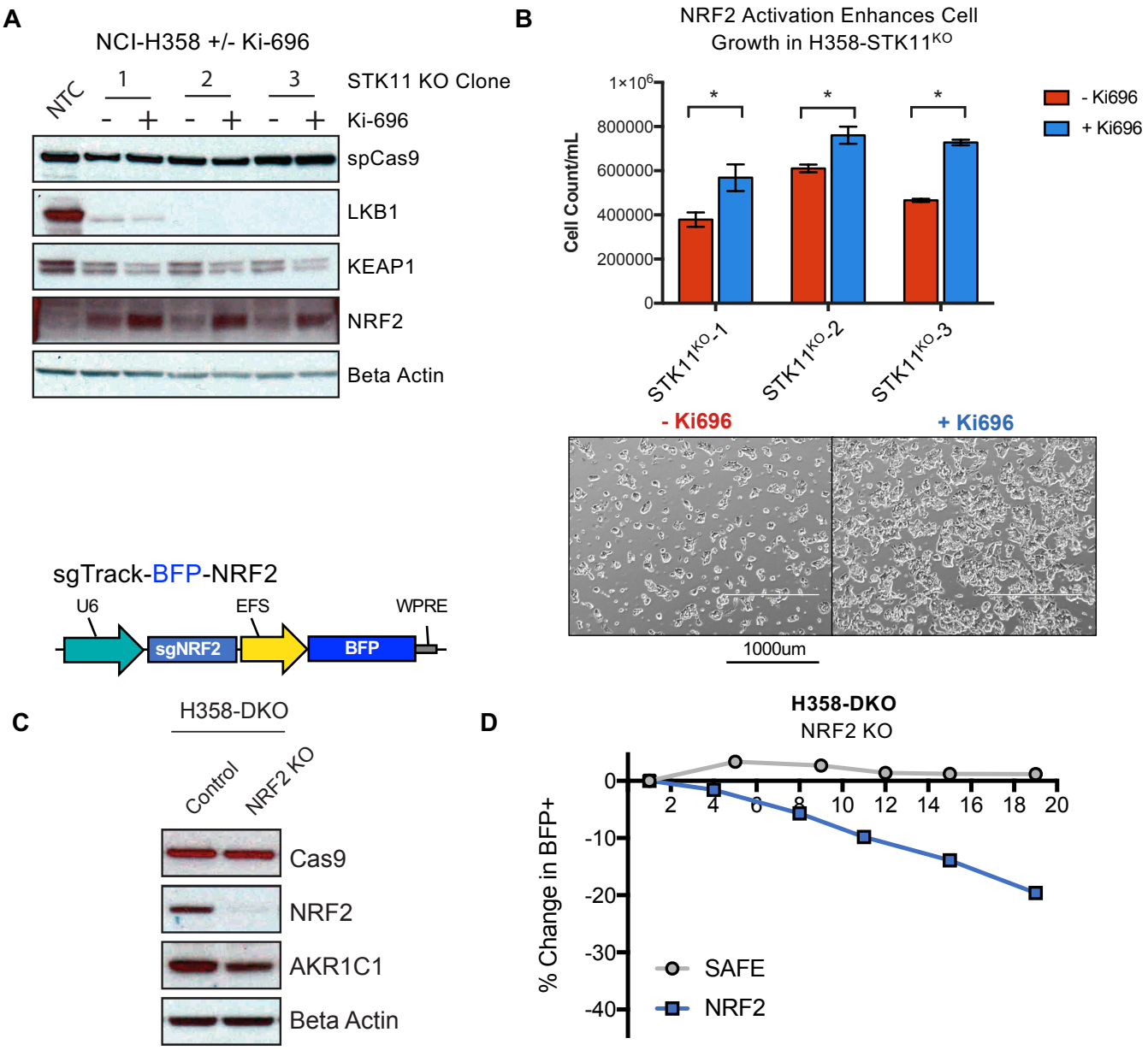
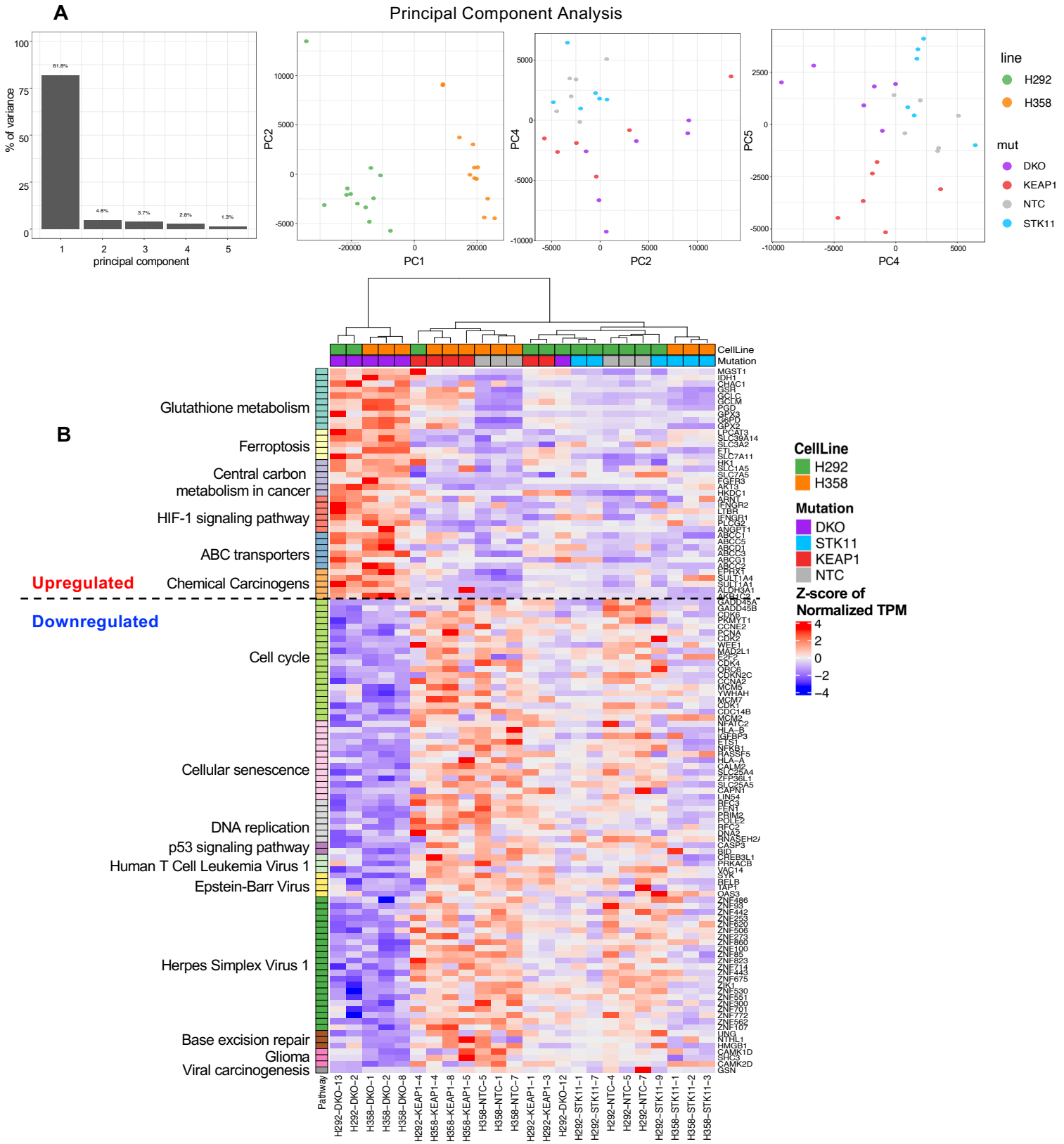


Supplementary Figure S1. *STK11* and *KEAP1* mutation landscape, Related to Figure 1 (A) Each dot on the lollipop plot indicates the locations of a genetic mutation present in a patient from the MSK Impact database. Plots compare specific mutations in patients with a single mutation in *KEAP1* or *STK11* to patients with *STK11/KEAP1* co-mutation. (B) Cancer cell fraction (CCF) for *KEAP1* (x-axis) and *STK11* (y-axis) are plotted for 292 samples. (C) Percentage of individuals among 4 mutational profiles (mutations in both *STK11* and *KEAP1*, *STK11* only, *KEAP1* only, or neither) with loss of heterozygosity (LOH) are shown in the opaque bars while the percentage of samples without LOH covering both genes are shown in transparent bars. The actual numbers and percent of each bar is annotated with in the bar. Fisher's exact test compared the enrichment of LOH occurring in co-mutants compared to *STK11* only patients (p-value = 8.4e-06, OR = 3.0), *KEAP1* only patients (p-value = 2.8e-07, OR = 3.5) and patients without a mutation in either gene (p-value < 2.2e-16, OR = 19.0).

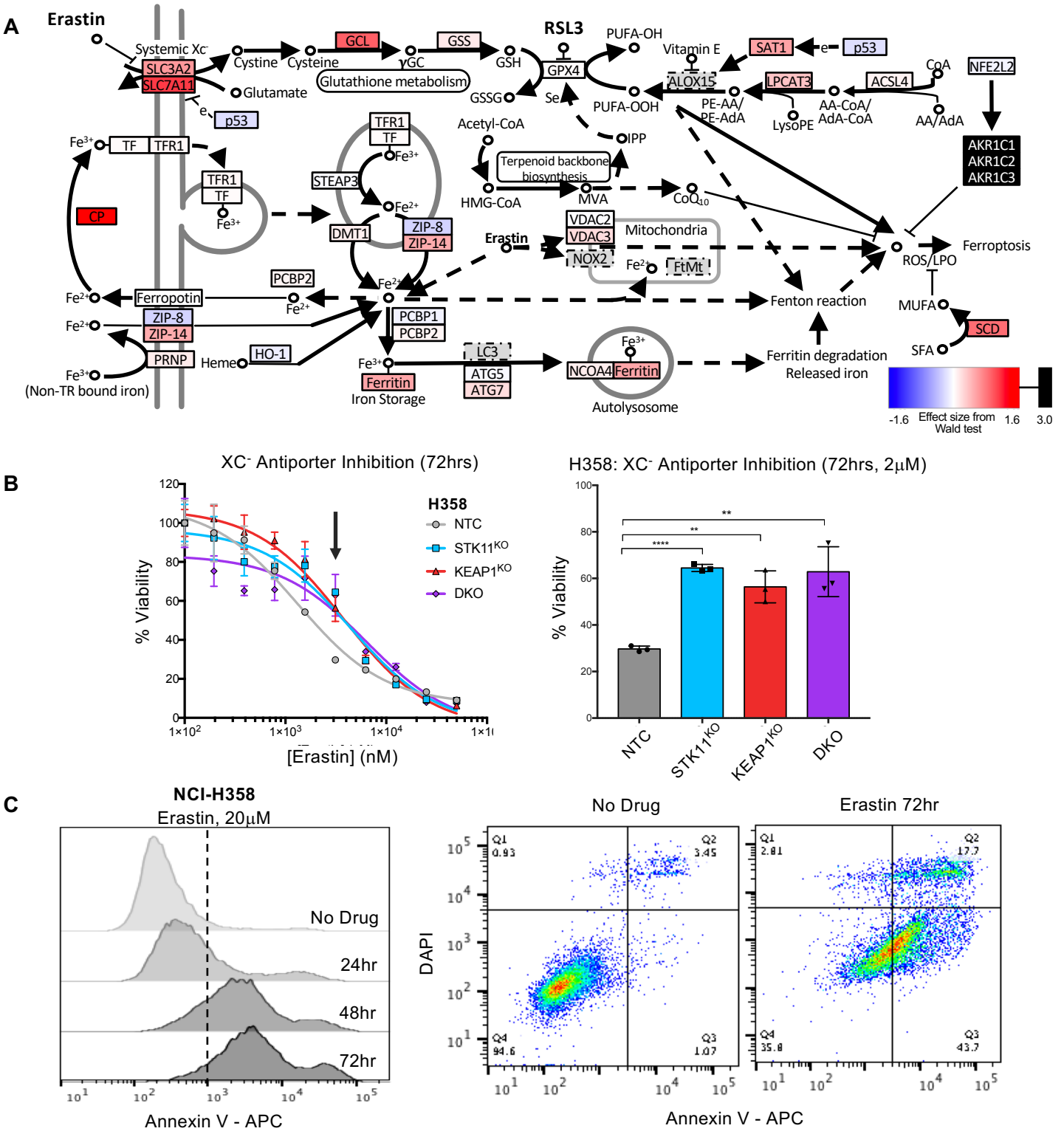


Supplemental Figure S2. NRF2 activation promotes cell growth in *STK11* mutant and NRF2 inhibition decreased cell growth in *STK11/KEAP1* co-mutant LUAD, Related to Figure 3

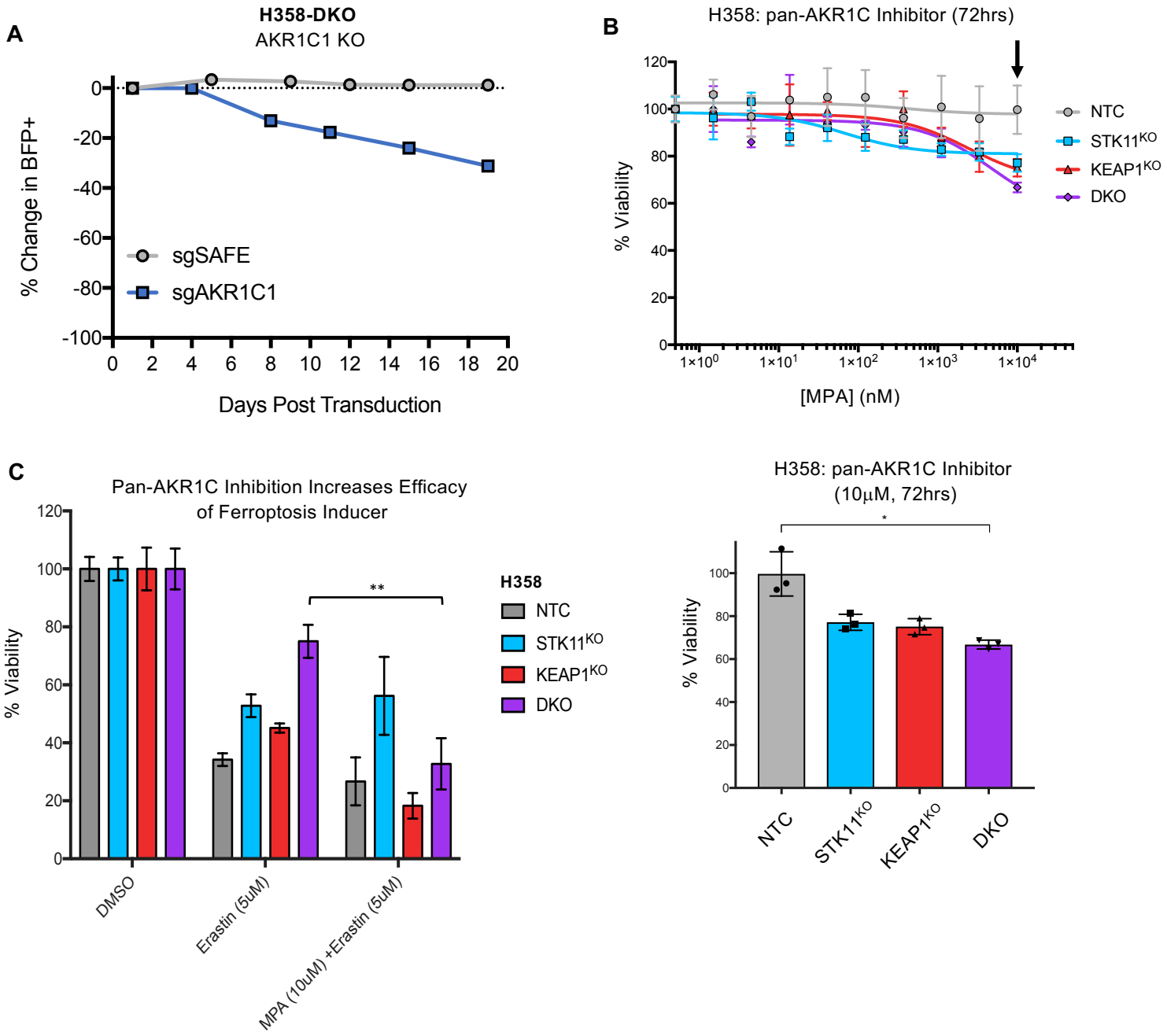
(A) Immunoblot of protein expression in H358-STK11^{KO} clones treated with Ki-696 (1μM) for 3 days. (B) Bar plot indicates number of cells per well treated with vehicle or Ki-696 (1μM) for 3 days. Microscope image was taken after 3 days in culture just before cells were counted. Significance was calculated by a two-sample test. *p<0.05; **p<0.01; ***p<0.001. (C) Vector map of lentiviral vector used to created NRF2 knockout in H358-DKO cells. Immunoblot of protein expression in H358-DKO cells and H358-DKO-NRF2^{KO} cells. (D) BFP Tracking experiment measures the change in percent of cells expressing the BFP-sgRNA vector over time. The safe targeting guide (grey) is the negative control targeting a non-coding region of the genome.



Supplementary Figure S3. *STK11/KEAP1* co-mutant LUAD has a distinct transcription profile, Related to Figure 4 (A) Principal component analysis based on RNA sequencing data groups clones first by cell line then by genotype. (B) Heatmap of top 116 differentially expressed genes between samples with double knockout versus others, across all cell lines and that are also found in 16 KEGG pathways. These KEGG pathways were significantly over-represented (q-value < 0.05) among genes that were either significantly higher or lower in double knock-out samples compared to others. Where genes are found in multiple pathways, they are assigned to the pathway with the most significant enrichment. Pathways are denoted on the left side of the plot while cell line and mutation status is shown on the top. Hierarchical clustering was performed using Manhattan distance and the Ward D method of clustering. Expression shown is a Z-score of the normalized TPM after removing the effect of cell line.

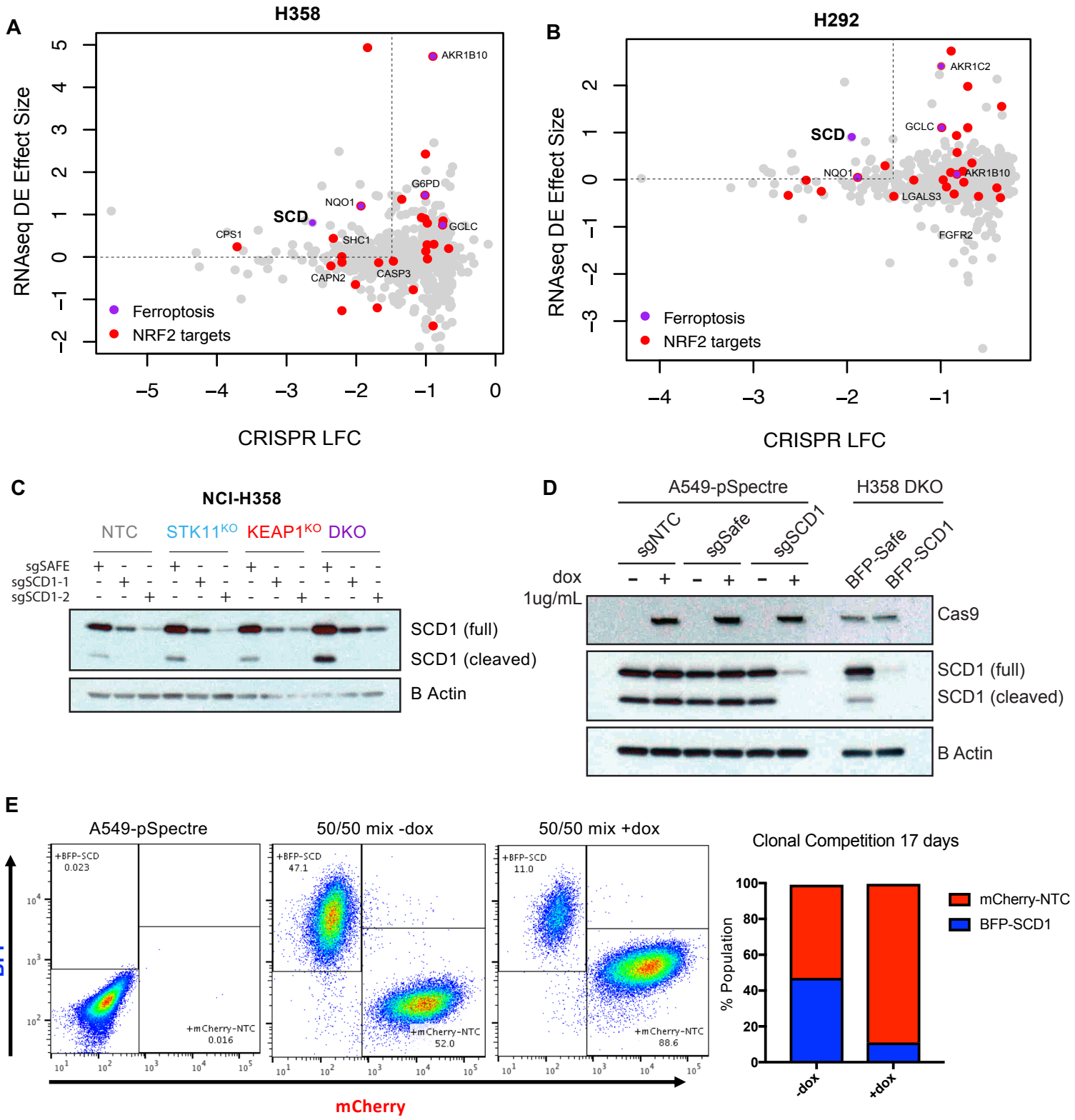


Supplementary Figure S4. Ferroptosis pathway is upregulated in *STK11/KEAP1* mutants and erastin treatment induces both ferroptosis and apoptosis, Related to Figure 4 (A) Genes in the ferroptosis pathway (as described in STAR methods) that are upregulated in *STK11/KEAP1* mutants compared to other genotypes are highlighted in red where the darkness of the color indicates the level of differential expression denoted by effect size from the Wald test. The effect size is the coefficient in the linear model used to assess differential expression and is analogous to the log fold change. Down-regulated genes are colored in blue. Genes shown in gray did not pass Sleuth basic thresholds of a minimum of 5 reads in at least 47% of samples. AKR1C1, AKR1C2 and AKR1C3 are shown in black because they exceed the scale shown (average effect size = 3.8). (B) Dose/response curve of H358 isogenic clones treated with the indicated dose of ferroptosis-inducer erastin for 72hrs. Arrow indicates the dose depicted in the bar graph with the greatest difference between genotypes. Significance was calculated by a two-sample t test. A Bonferroni correction was performed across the six tests so that *p<0.008; **p<0.002; ***p<0.0002. (C) Annexin V staining in H358 cells treated with erastin at 20uM for the indicated time period. A shift to the right indicates an increase in Annexin V staining denoting apoptosis. DAPI positive cells (right) are considered dead while Annexin V positive DAPI negative cells are undergoing apoptosis.

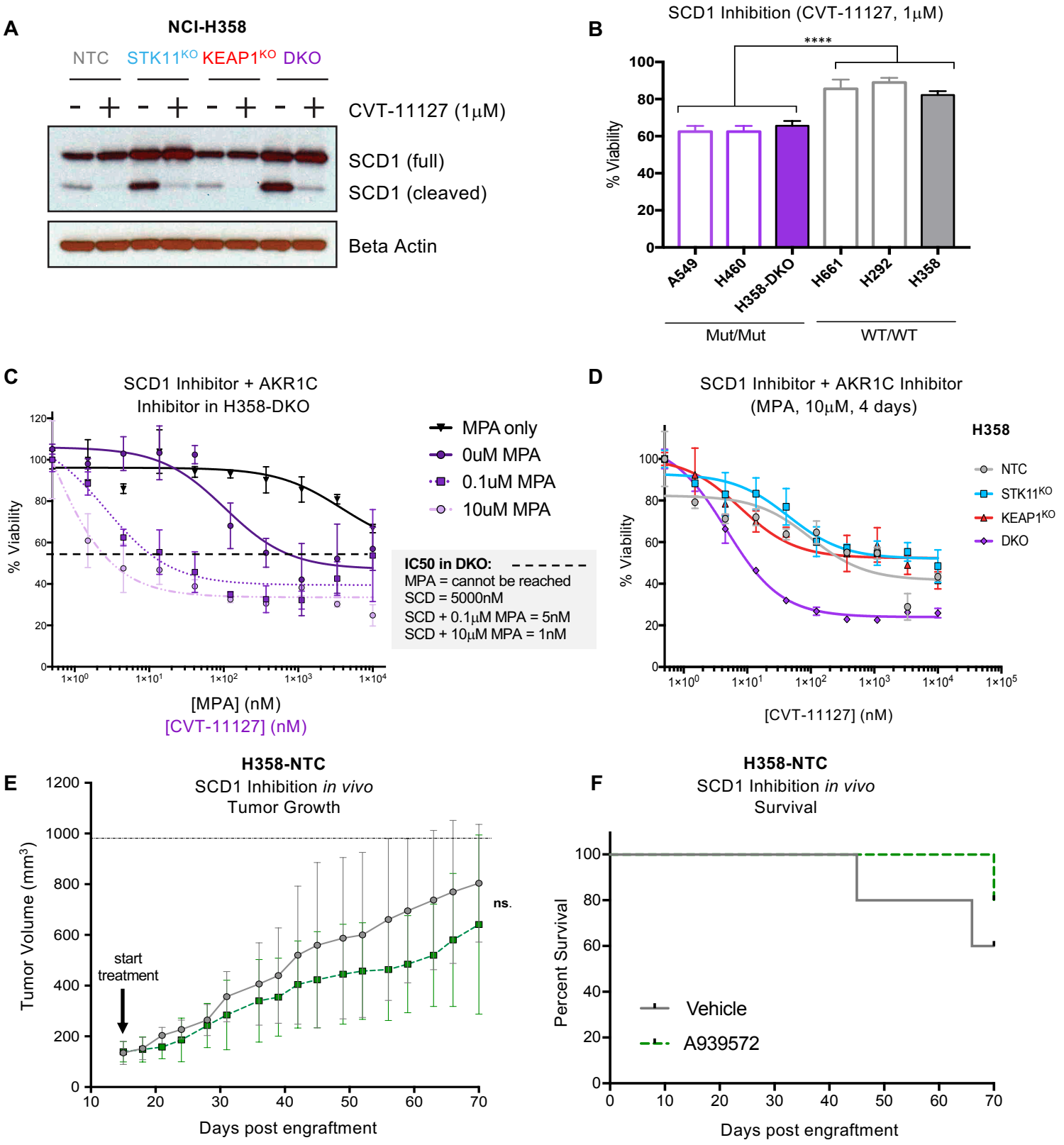


Supplementary Figure S5. AKR1C inhibition has limited efficacy *in vitro* but shows improved response in combination with ferroptosis inducer erastin, Related to Figure 5

(A) BFP Tracking experiment measures the change in percent of cells expressing the BFP-sgRNA vector over time. The safe targeting guide (grey) is the negative control targeting a non-coding region of the genome. (B) Dose/response curve for H358 isogenic clones treated with the indicated dose of pan-ARK1C inhibitor Medroxyprogesterone 17-acetate (MPA) for 72 hours. Arrow indicates dose shown in the bar graph below. Significance was calculated by a two-sample t test between samples at either end of the bracket. A Bonferroni correction was performed across the six tests so that *p<0.008; **p<0.002; ***p<0.0002. (C) Viability measurement for H358 isogenic clones treated with 10μM of MPA alone, 5μM of erastin alone, or a combination of 10μM MPA and 5μM erastin for 72 hours. Significance was calculated by a two-sample test between DKO samples where *p<0.05; **p<0.01; ***p<0.001.



Supplementary Figure S6. SCD1 is identified in CRISPR/Cas9 Screen and RNAseq, Related to Figure 6 (A) Differential sgRNA abundance between DKO and NTC is denoted by genewise log fold changes (LFC) plotted as the mean of the statistically significant sgRNAs for each gene on the x axis. A cutoff of -1.5 LFC was chosen to identify candidate hits from the two screens. RNAseq betas value calculated by Sleuth was plotted on the y axis to identify hits that were transcriptionally upregulated in H358 and (B) H292. Genes in the ferroptosis and NRF2 KEGG pathways were highlighted. (C) Immunoblot confirms knockout of SCD1 in H358 isogenic clones used for BFP tracking experiment in Figure 6E. (D) Immunoblot confirms dox-inducible knockout of SCD1 in A549 cells used for clonal competition experiment relative to protein expression in H358-DKO and H358-DKO-SCK1^{KO}. (E) Clonal competition assay measures change in representation of A549 cells expressing BFP and a targeted guide RNA to SCD1 or expressing mCherry and a non-targeting control guide. A549 cells express dox-inducible Cas9 where -dox cells are Cas9 negative and +dox cells are Cas9 positive.



Supplementary Figure S7. SCD1 inhibition alone and in combination with AKR1C inhibition, Related to Figure 5 and 7 (A) Immunoblot of SCD1 protein expression in H358 isogenic clones treated with CVT-11127 at 1μM for 24 hours. (B) Viability measured across three *STK11/KEAP1* co-mutant cell lines compared parental H358 cell line (WT/WT) and two WT/WT cell lines H661 and H292 treated with SCD1 inhibitor CVT-11127 at 1μM for 4 days. Significance was calculated by a two-sample t test comparing mut/mut group to wt/wt group where *p<0.05; **p<0.01; ***p<0.001, ****p<0.0001. (C) Dose-response measurements for H358-DKO clone treated with a titration of MPA alone (black curve), a titration of CVT-11127 alone (solid purple) or a combination of MPA at the indicated dose plus titration of CVT-11127. The black horizontal dotted line indicates IC50 and IC50 concentrations are listed in the table to the right. (D) Dose-response measurements across H358 isogenic cell lines treated with 10μM of AKR1C inhibitor (MPA) and a titration of SCD1 inhibitor (CVT-11127) for 4 days. (E) Tumor volume of H358-NTC tumors treated with vehicle (grey) or 50mg/kg SCD1 inhibitor A939572 (dotted green). (F) Survival data of mice from (E) where survival was denoted as time from injection of cells to tumor volume of 1000mm^3.

Overall Demographics

Characteristic	N=1235
Age	65 (57, 72)
Smoking	
Ever	851 (69%)
Never	384 (31%)
Sex	
Female	737 (60%)
Male	498 (40%)
KRAS	359 (29%)
STK11	209 (17%)
KEAP1	208 (17%)
CoMutants	124 (10%)

Double Mutant Demographics

Characteristic	0, N=1111	1, N=124	p-value*
Age	64 (57, 72)	68 (60, 76)	<0.001
Smoking			<0.001
Ever	730 (66%)	121 (98%)	
Never	381 (34%)	3 (2.4%)	
Sex			0.016
Female	676 (61%)	61 (49%)	
Male	435 (39%)	63 (51%)	
KRAS	293 (26%)	66 (53%)	<0.001

* Statistical test performed: Wilcoxon rank-sum test; chi-square test of independence

Table S5: Patient Characteristics, Related to Figure 1