Supplemental Materials and Methods

Generation of shCERK lentivirus and stable *CERK* downregulation in H838 and H358 cells

shRNAs targeting CERK in pLKO.1 vector were obtained from Dharmacon. shCERK_1 (RHS3979-201766551) and shCERK4 (RHS3979-201766554) were selected based on CERK1 knock-down efficiency. To produce virus, 293T cells were transfected with pLKO.1 Vector, shCERK_1 or shCERK4 together with psPAX2 (addgene #12260) and pMD2.G (addgene #12259) using Polyethylenimine (PEI, Sigma). The supernatant of the 293T cells was harvested at 48 and 72hr post-transfection, passed through a 0.45 μm filter and concentrated by centrifugation utilizing lenti-X concentrator (Takara). H838 and H358 lung cancer cells were transduced with viral supernatant and selected with puromycin (2ug/ml) for 2-3 days as described (27).

Senescence assay

Cell senescence was evaluated by using Senescence Detection Kit (Sigma Aldrich) following manufacturer's protocol. Briefly, cells seeded in 12-well plates were washed with 1X PBS, fixed in Fixation Solution at room temperature for 15 minute, then washed with 1X PBS and placed in Staining Solution Cells at 37^{o}C overnight. Cells were observed under a microscope. 5 random images were captured each well and the number of cells with increased β-galatosidase (β-gal) activity or blue stained cells (senescent cells) in each image were counted. Senescence value in each well is the average of β-gal positive cells from 5 random images captured from each well.

Plasmid transfection

Cells were transfected with plasmids expressing V5-tagged CERK using Lipofectamine 2000 Reagent (Invitrogen) following manufacturer's protocol. After 24 hours of transfection, cells were harvested for Western immunoblotting or further used in clonogenic survival assay.

Supplemental tables and figures

Supplemental Table S1: List of drugs/reagents used to treat NSCLC cells. NSCLC cells were treated with reagents/drugs in serum-free RPMI medium.

Drugs/reagents	Company
BEZ235 (AKTi)	Sigma
Cisplatin (Cis)	Sigma
Cloroquine (CQ)	Sigma
Colchicine (Col)	Sigma
Cloroquine (CQ)	Sigma
Deferoxamine (DF)	Sigma
Erastin	Selleckchem
Ferrostatin-1 (Fer-1)	Sigma
N-acetyl-L-cysteine (NAC)	Sigma
NVP-231	Cayman
Z-vad-fmk (Z-vad)	Selleckchem

Supplemental Table S2: List of antibodies used as primary antibodies in Western immunoblotting.

Antibody	Company
Cleaved PARP	Cell Signaling Technology
β-actin	Cell Signaling Technology
TFR1	Cell Signaling Technology
HSP90	Cell Signaling Technology
VDAC1	Cell Signaling Technology
VDAC2	Cell Signaling Technology
P-AKT	Cell Signaling Technology
AKT	Cell Signaling Technology
Phospho (S/T) -AKT substrate	Cell Signaling Technology
β-tubulin	Cell Signaling Technology
COXIV	Cell Signaling Technology
Bcl2	Santa Cruz Biotechnology
BclxL	Cell Signaling Technology
LC3a/β	Cell Signaling Technology
Caspase-9	Cell Signaling Technology
ACO2	Cell Signaling Technology
Bax	Cell Signaling Technology
V5	Invitrogen

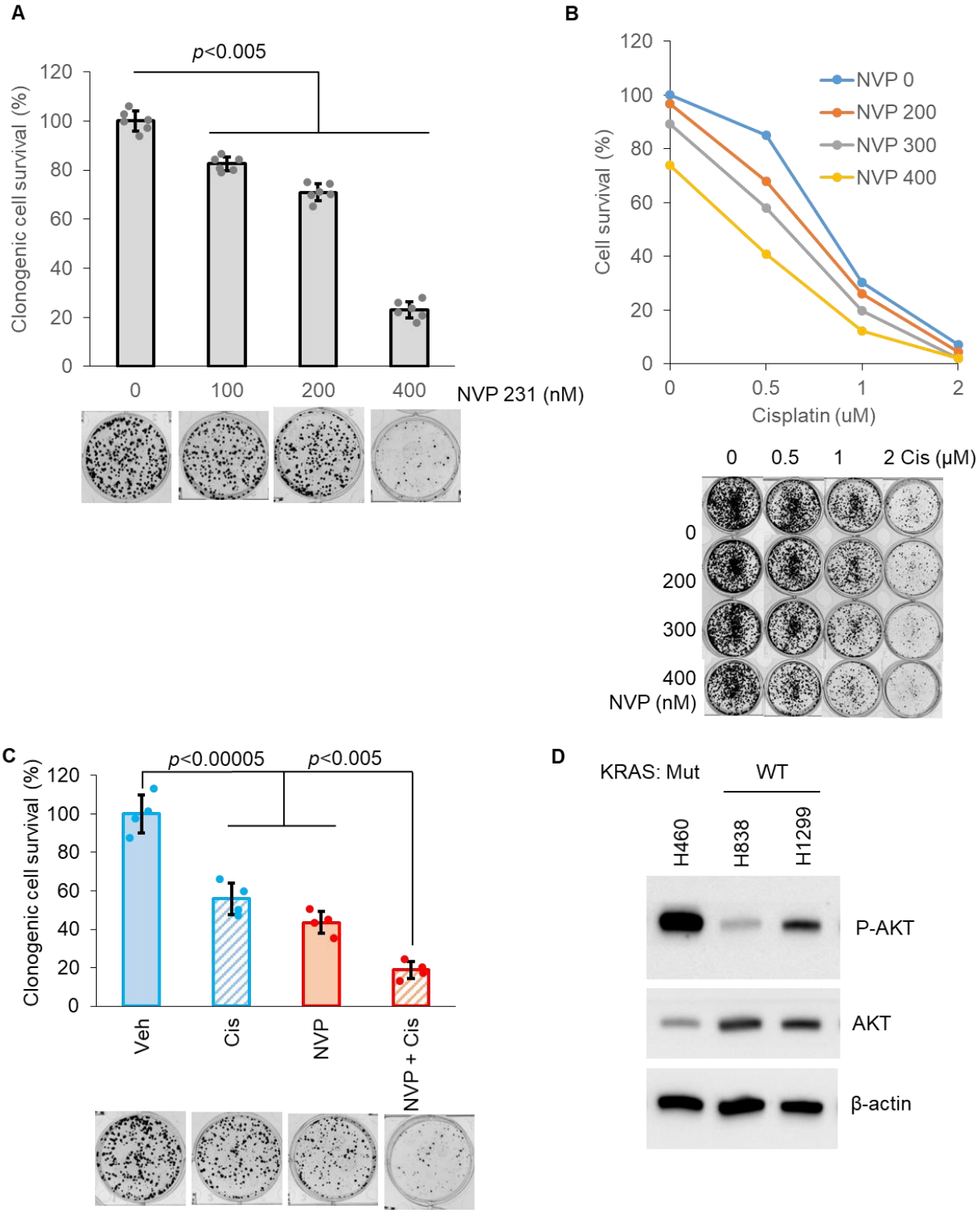
Supplemental Table S3: Genetic background of NSCLC cell lines.

NSCLC cell line	Genotype						Histology
	KRAS	*NRAS*	*STK11 (LKB1)*	*CDKN2a*	*SMARCA4 (BRG1)*	*p53*	
A549	mut	WT	mut	mut	mut	WT	NSCLC (Adenocarcinoma)
H358	mut	WT	WT	WT	WT	null	NSCLC (Bronchioloalveolar carcinoma)
H460	mut	WT	mut	mut	WT	WT	NSCLC (Large cell carcinoma)
H1792	mut	WT	WT	WT	WT	mut	NSCLC (Adenocarcinoma)
H838	WT	WT	mut	WT	WT	WT	NSCLC (Adenocarcinoma)
H1299	WT	mut	WT	WT	WT	null	NSCLC (Large cell carcinoma)

Supplemental Table S4: Half-maximal inhibitory concentration (IC_{50}) of NVP-231 in NSCLC cells. NSCLC cells were treated with varying dose of NVP231 (0-5000 nM) for 48 hours and then cells were utilized in clonogenic cell survival assay (n=4 from 2 independent occasions). Clonogenic cell survival data (average value) were used to calculate IC_{50} values.

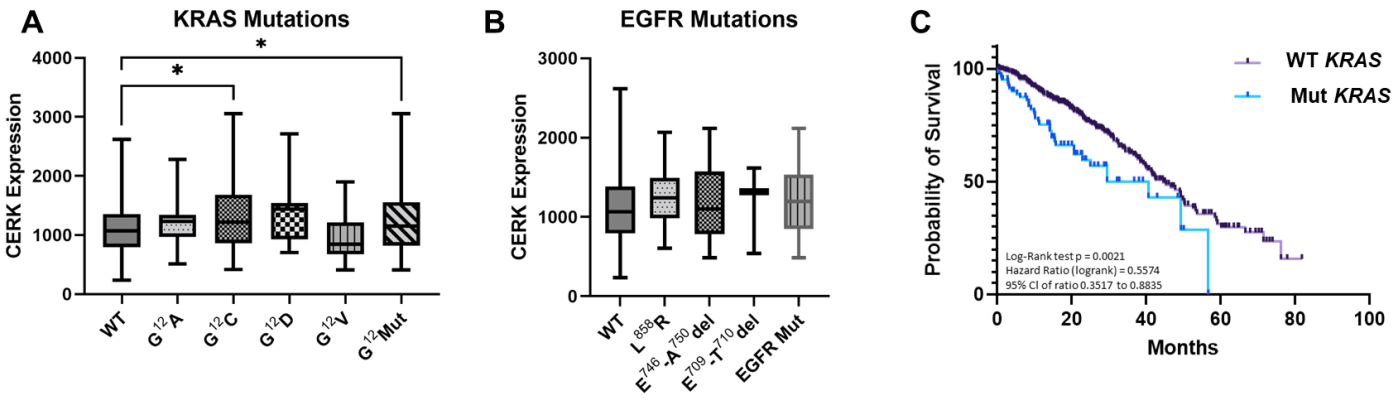
NSCLC cell line	IC_{50}	Histology
A549	210	NSCLC (Adenocarcinoma (LUAD))
H358	200	NSCLC (Bronchioloalveolar carcinoma (BAC))
H460	406	NSCLC (Large cell carcinoma (LCC))
H1792	402	NSCLC (Adenocarcinoma (LUAD))
H1299	833	NSCLC (Adenocarcinoma (LUAD))
H838	1248	NSCLC (Large cell carcinoma (LCC))

Supplemental Figure S1: NSCLC cells with a *KRAS* mutation (H460) are more susceptible to CERK inhibition and have higher AKT phosphorylation, ferroptosis induction and cisplatin responsiveness. (A) H460 cells (Large Cell Carcinoma Histology (LCC)) were treated with vehicle or NVP231 (100-400 nM) in serum-free media for 48 hours then utilized in clonogenic survival assay. (B) Crystal violet cell survival assay were performed from H460 cells treated with NVP 231 (100-400 nM) for 8 hours followed by 16-hour cisplatin treatment. (C) Clonogenic cell survival assay were undertaken from NSCLC cells treated with NVP 231 (400 nM) for 8 hours then 16-hour treatment of cisplatin (0.5 μM). All treatment were performed in serum-free media. (D) H460/H838/H1299 cells at approximately 40-50% confluency were placed in serum-free media for 16 hours, then cell lysates were prepared and utilized in SDS-PAGE/immunoblotting (C). Data in (A) and (C) are means ± SD; n = 4-6 from two independent occasions. Data in (B) are mean. Unless otherwise noted by a p-value, data are not significant between a depicted or non-depicted group comparison.

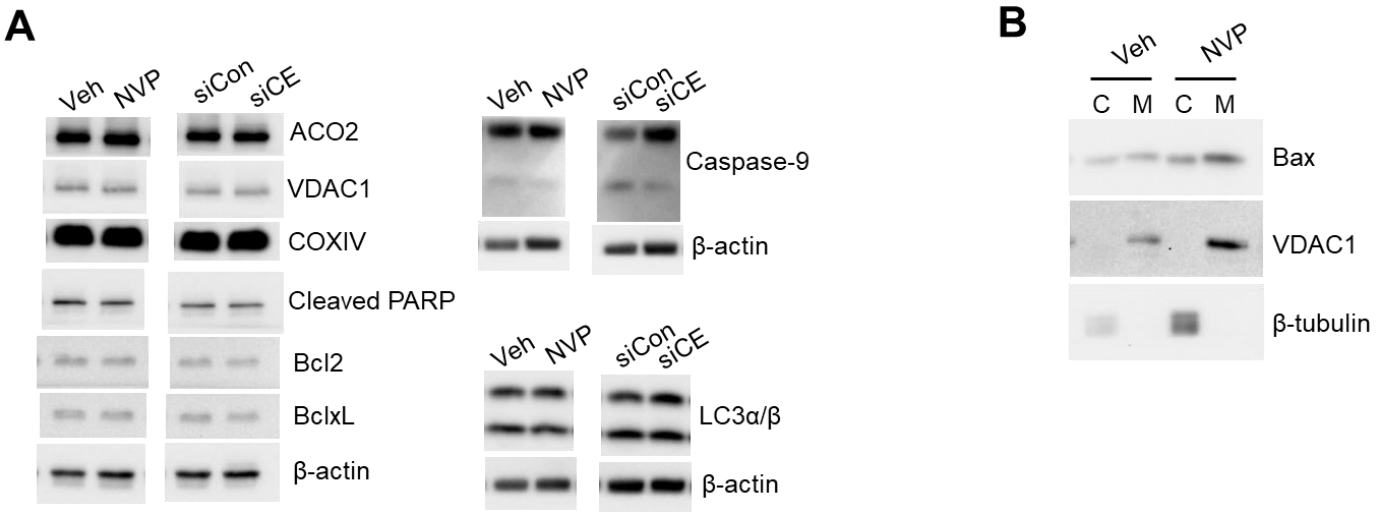

A
p<0.005
Clonogenic cell survival (%)
120
100
80
60
40
20
0
0
100
200
400
NVP 231 (nM)
B
Cell survival (%)
NVP 0
NVP 200
NVP 300
NVP 400
0
0.5
1
2
Cisplatin (uM)
0
0.5
1
2 Cis (μM)
0
200
300
400
NVP (nM)
C
p<0.00005
p<0.005
Clonogenic cell survival (%)
Veh
Cis
NVP
NVP + Cis
D
KRAS: Mut
WT
H460
H838
H1299
P-AKT
AKT
β-actin

Supplemental Figure S2: (A) Lung adenocarcinoma patients with G12 activating mutations have increased CERK expression. Comparison of CERK expression in human lung adenocarcinoma patients harboring WT KRAS ver G12 KRAS activating mutations; Data are demonstrated as means ± SD with n = 436 for WT, 15 for G12A, 62 for G12C, 18 for G12D, 30 for G12V, and 128 for all G12 activating mutants combined (G12Mut). *p<0.05 and unless otherwise noted by an * or depicted p-value, data are not significant between depicted groups; (**B**) Lung adenocarcinoma patients with EGFR mutations have normal CERK expression compared to patients with WT EGFR. Comparison of CERK expression in human lung adenocarcinoma patients harboring WT EGFR ver EGFR mutations; Data are demonstrated as means ± SD with n = 436 for WT, 18 for $L^{858}R$, 15 for E^{746}-A^{750}del, 3 E^{709}-A^{710}del, and 36 for all EGFR mutants combined (EGFR Mut). Unless otherwise noted by a p-value or *, data are not significant between depicted groups. (**C**) Comparison of survival rate between lung cancer patients with WT versus Mut *KRAS*. TCGA PanCancer Atlas LUAD Patient survival data was collected through CBioPortal and divide into two groups, patients harboring WT *KRAS* and patients harboring Mut *KRAS* (G12 Mut) and analyzed for survival probability. Data are represented with a Kaplan Meier plot, n = 342 for WT patients and 94 for Mut *KRAS* patients.

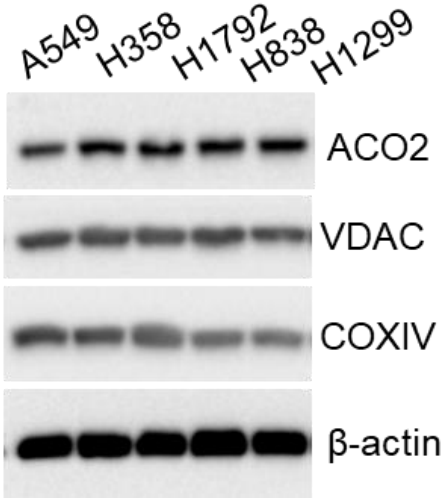
Supplemental Figure S2



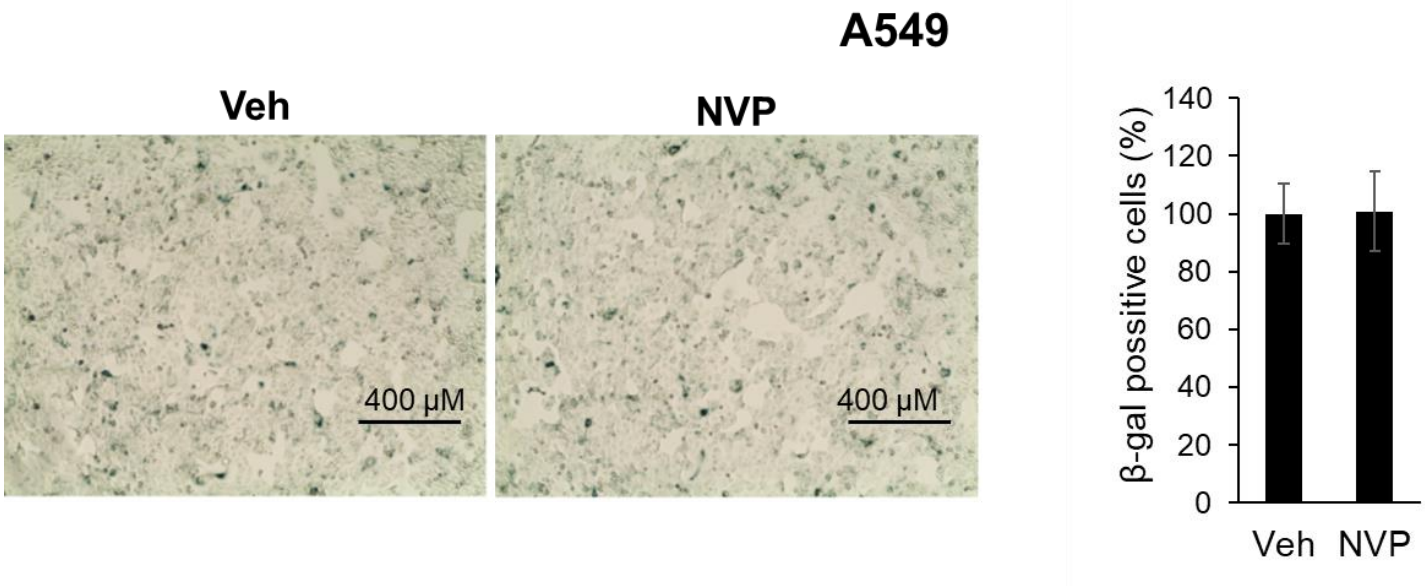
Supplemental Figure S3: Inhibition/Down-regulation of CERK does not have effect on mitochondria, apoptosis-related and LC3 protein level. A549 cells were treated with vehicle (Veh) or NVP 231 (400 nM) for 24 hours or transfected with control or CERK siRNA (siCon or siCE) for 48 hours. (A) Protein lysates were then prepared and subjected to SDS-PAGE/immunoblotting. (B) Mitochondrial (M) and cytosolic (C) protein lysate was prepared and subjected to SDS-PAGE/immunoblotting.



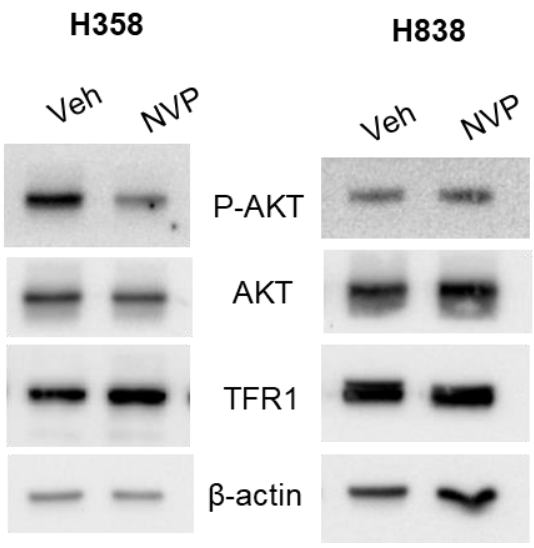
Supplemental Figure S4: Expression of mitochondria proteins in NSCLC cells. A549/H358/H1792/H838/H1299 cells (all are LUAD except H1299 cells, which are LCC) at approximately 40-50% confluency were placed in serum-free media for 16 hours, and then cell lysates were prepared and subjected to SDS-PAGE/immunoblotting.



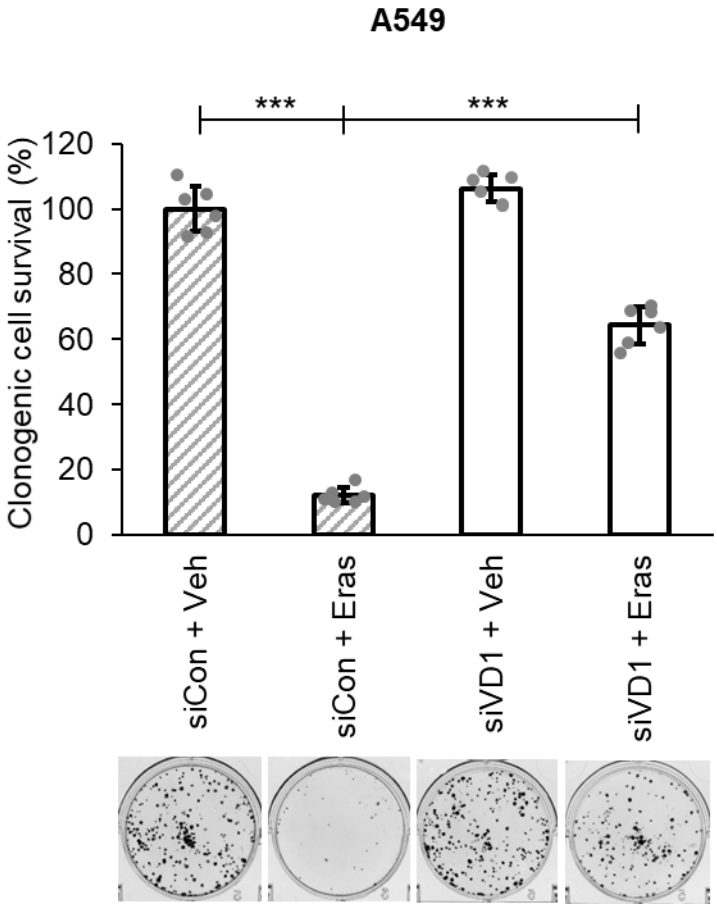
Supplemental Figure S5: Inhibition of CERK does not affect cellular senescence. A549 cells (lung adenocarcinoma (LUAD) histology) were treated with vehicle (Veh) or NVP 231 (400 nM) for 24 hours and then subjected to senescence assay. Data are mean ± SD; n = 6 from two independent occasions. Scale bar = 400 µm. Unless otherwise noted by an * or depicted p-value, data are not significant between a depicted or non-depicted group comparison; p > 0.05.



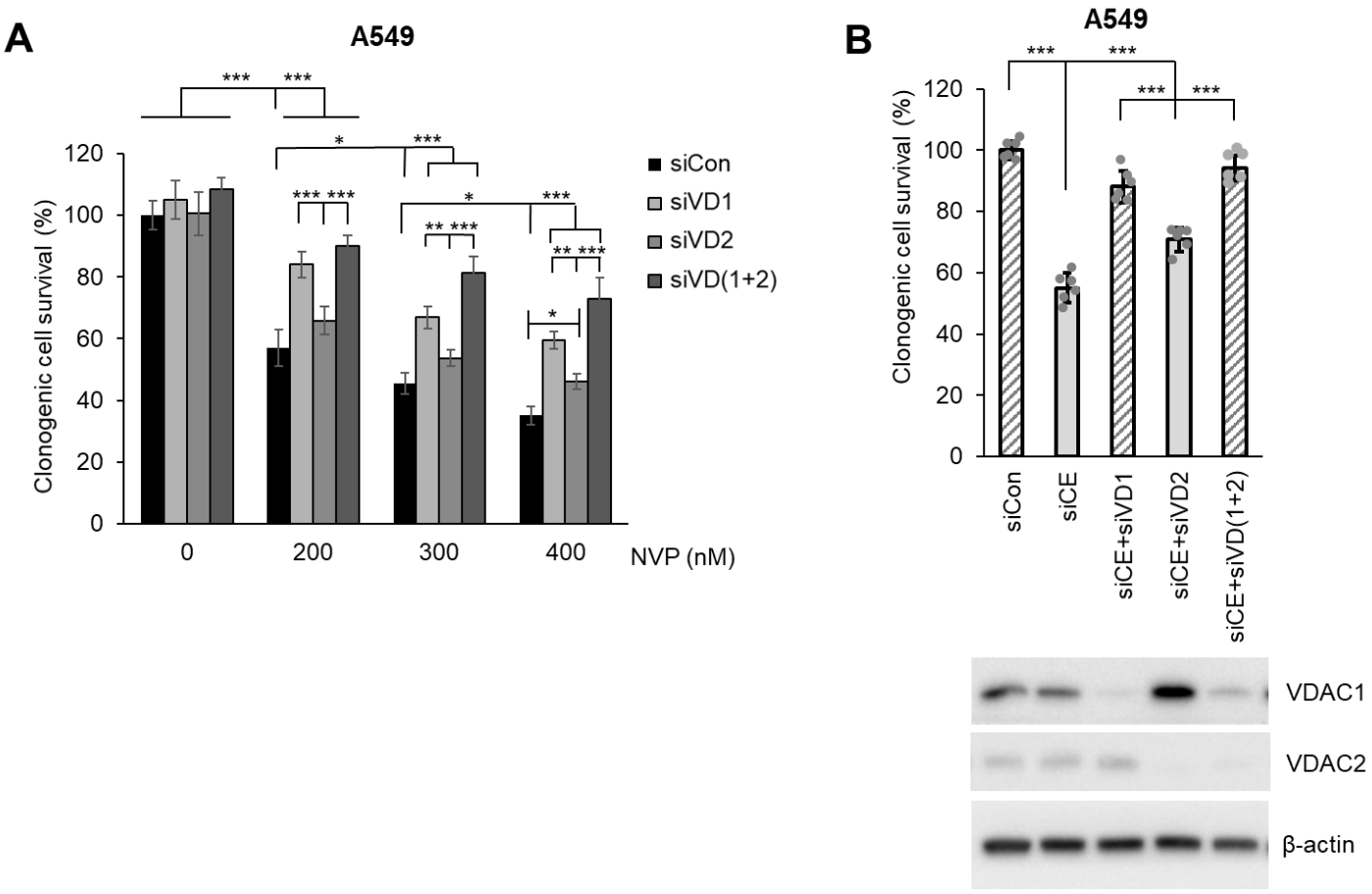
Supplemental Figure S6: Inhibition of CERK attenuates AKT phosphorylation and ferroptosis in Mut but not in WT *KRAS* NSCLC cells. H358 (Mut *KRAS*) cells ((BAC-derived) or H838 (WT *KRAS*) cells (LUAD-derived) were treated with NVP 231 (400 nM) and subjected to SDS-PAGE/immunoblotting.



Supplemental Figure S7: VDAC1 is important for erastin-induced ferroptosis. A549 cells (LUAD) transfected with control/VDAC1 siRNA (siCon, siVD1) for 48 hours were treated with vehicle (Veh) or erastin (Eras, 10 μM) for 24 hours before the cells were utilized in clonogenic survival assay. Data in graph are means ± SD; n = 6 from two independent occasions; ***p < 0.0001. Unless otherwise noted by an * or depicted p-value, data are not significant between a depicted group comparison; p > 0.05.

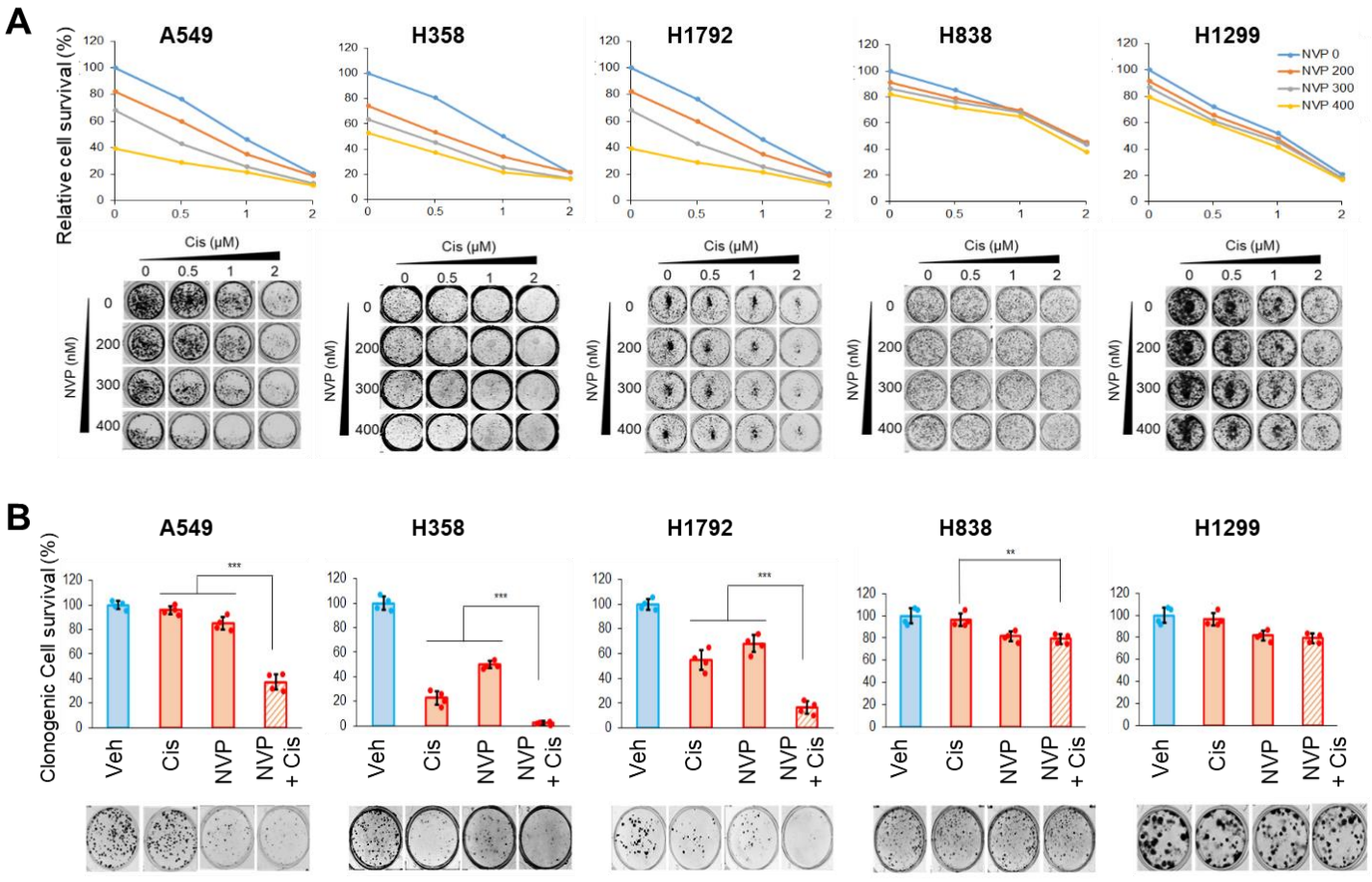


Supplemental Figure S8: The role of VDAC2 in CERK-mediated cell survival enhancement in Mut *KRAS* NSCLC cells. (**A**) A549 cells (LUAD) were transfected with control/VDAC1/VDAC2/both VDAC1 and VDAC2 siRNA [siCon, siVD1, siVD2, siVD(1+2)] for 48 hours then with NVP-231 (0-400 nM) for 24 hours. (**B**) A549 cells were transfected with siCon, siVD1, siVD2 or siVD(1+2) for 24 hours then with control/CERK siRNA (siCon, siCE) for additional 48 hours. Cells in (A) and (B) were subjected to clonogenic cell survival assay or Western immunoblotting. Data in graphs are means ± SD; n = 6 from two independent occasions. *p<0.05, **p<0.005, *** p<0.0005. Unless otherwise noted by an * or depicted p-value, data are not significant between depicted groups; p > 0.05.

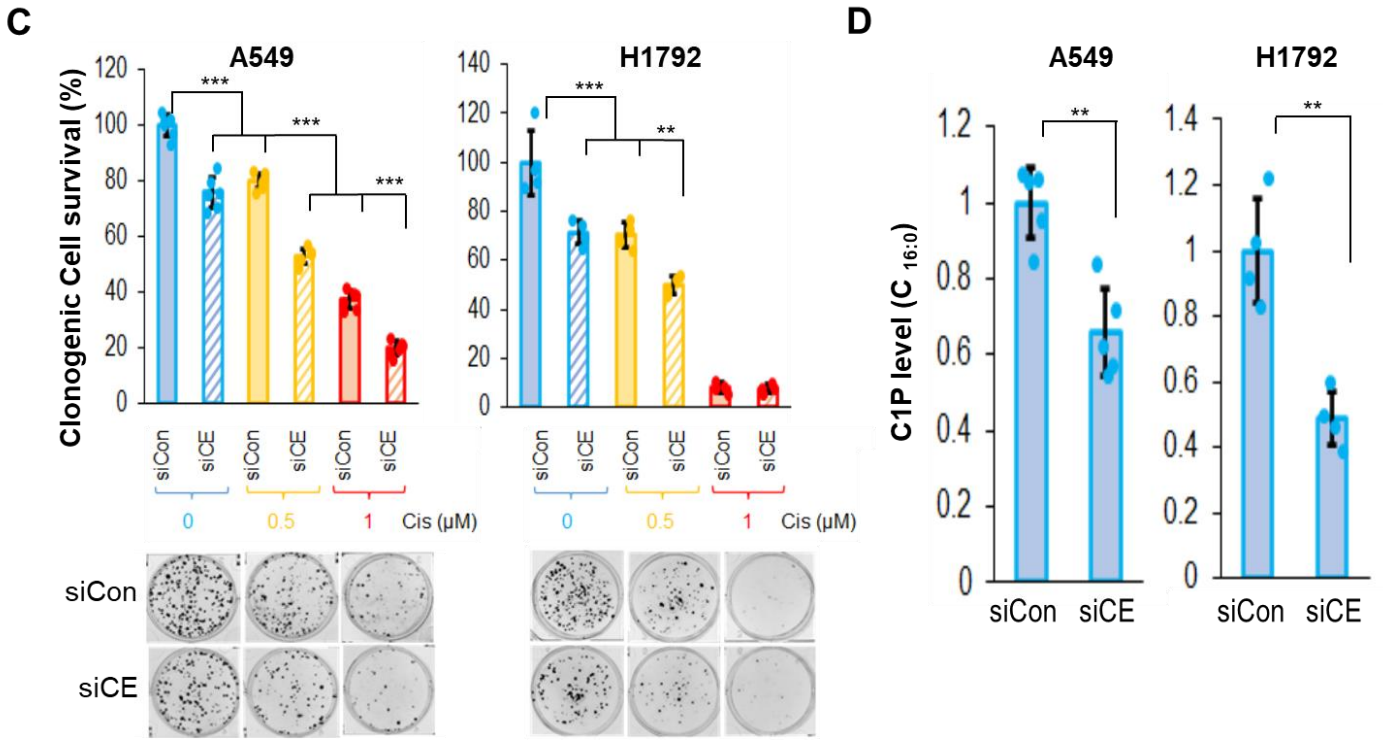


Supplemental Figure S9: Ceramide kinase inhibition/downregulation sensitizes Mut *KRAS* NSCLC cells to cisplatin treatment. (**A**) Cell survival assay was performed on Mut *KRAS* (A549 (LUAD), H358 (BAC) and H1792 (LUAD)) or WT *KRAS* (H838 (LUAD) and H1299 (LCC)) cells treated with NVP 231 (0-400 nM) for 8 hours followed by 16-hour cisplatin (Cis, 0-2 µM) treatment. (**B**) Clonogenic cell survival assay was undertaken from NSCLC cells treated with vehicle (Veh) or NVP 231 (400 nM) for 8 hours then 16-hour treatment of Veh or Cis (0.5 µM). (**C**, **D**) A549/H1792 cells transfected with (siCon) or CERK (siCE) for 48 hours were utilized in clonogenic cell survival assay (C) or mass spectrometry analysis for $C_{16:0}$ C1P level (D). Data in (A) are means; data in (B-D) are means ± SD; n = 4-6 from at least two independent occasions in (A-D); **p<0.005, ***p<0.0005. Unless otherwise noted by an * or depicted p-value, data are not significant between depicted groups; p > 0.05.

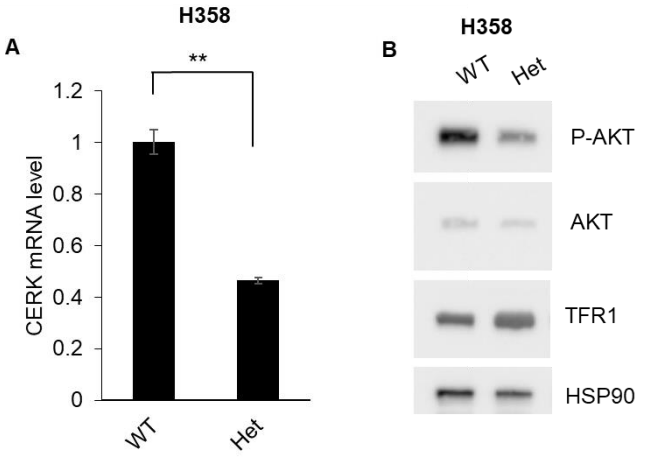
Supplemental figure S9.

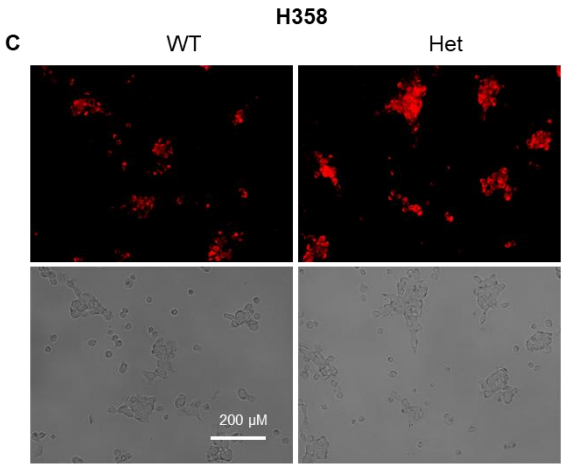


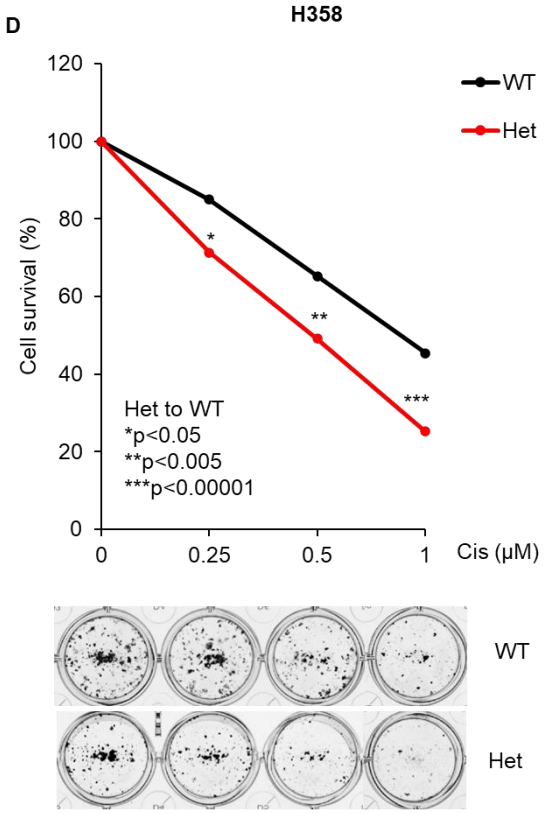
Supplemental figure S9 continued

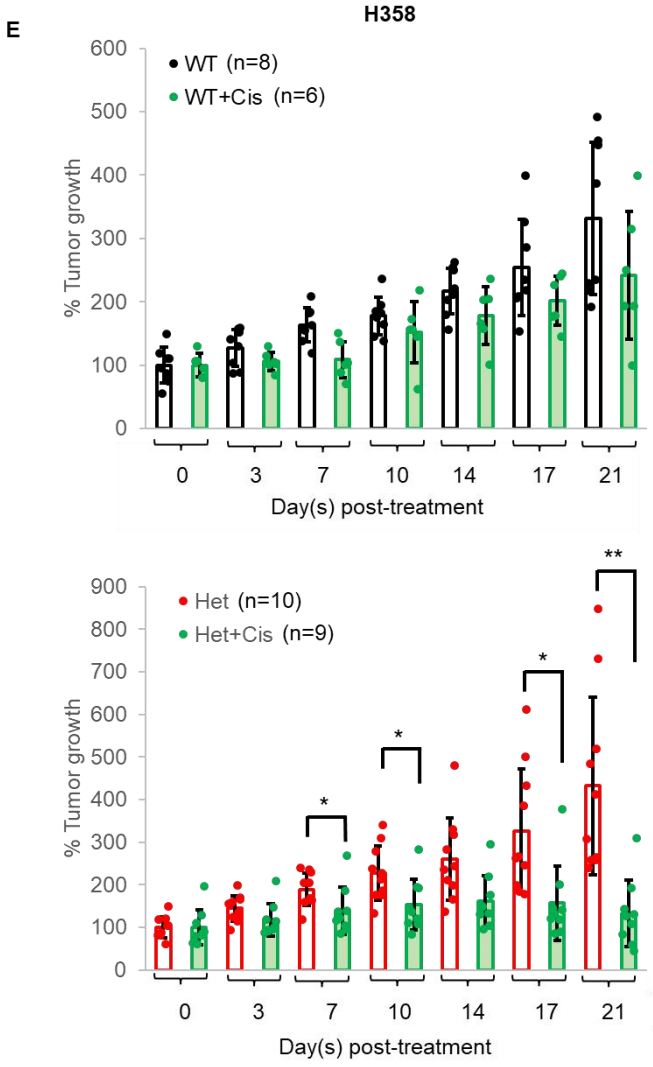


Supplemental Figure S10: Comparison of CERK expression, AKT phosphorylation, ferroptosis induction, MMP, cell survival and cisplatin responsiveness level in heterozygous CERK versus WT H358 cells. Heterozygous CERK (CERK Het) or WT cells (H358 (BAC)) were grown to reach approximately 40-50% confluency. (A) RT-qPCR for CERK were undertaken from RNAs samples prepared from cell lysates. (B) Cell lysates were subjected to Western immunoblotting. (C) Cells were subjected to MMP assay. (D) Cells were treated with cisplatin for 24 hrs before clonogenic survival assay were undertaken. (E) H358 cells with CERK WT (+/+) or Het (+/-) were injected into the flanks of nude mice (10^6 cells/mice). Mice developing tumors with approximate 100 mm^3 volume were treated with cisplatin (3mmg/kg body weight) twice/week for 3 weeks. Tumor size (volume) was measured twice/week. Data in (A) and (E) are means ± SD; data in (D) are mean; n = 4 and **p<0.0001, in (A) and n=8 in (D) from two independent occasions. n = 1 tumor. Scale bar = 200 µM. For Panels D and E, *p<0.05, **p<0.005, *** p<0.0001. Unless otherwise noted by an * or depicted p-value, data are not significant between depicted groups; p > 0.05.

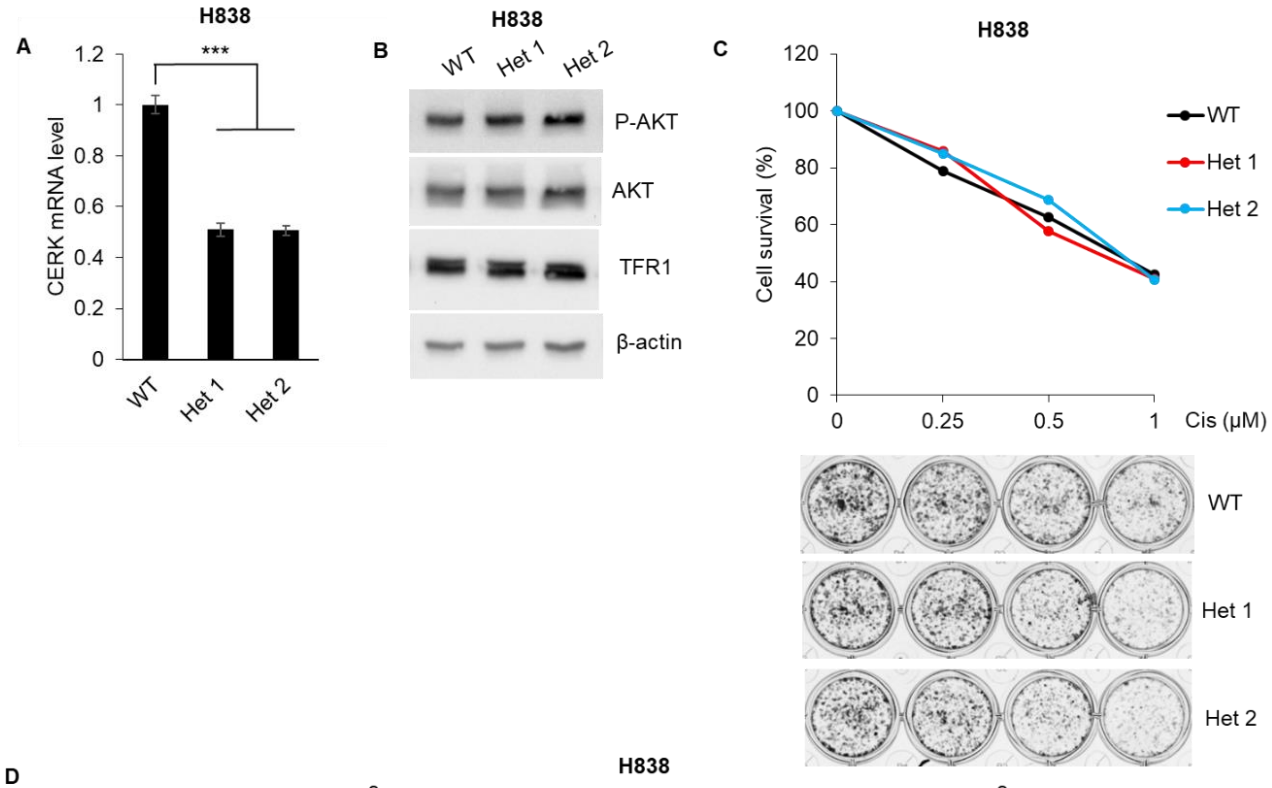

A
H358
**
CERK mRNA level
1.2
1
0.8
0.6
0.4
0.2
0
WT
Het
B
H358
WT
Het
P-AKT
AKT
TFR1
HSP90

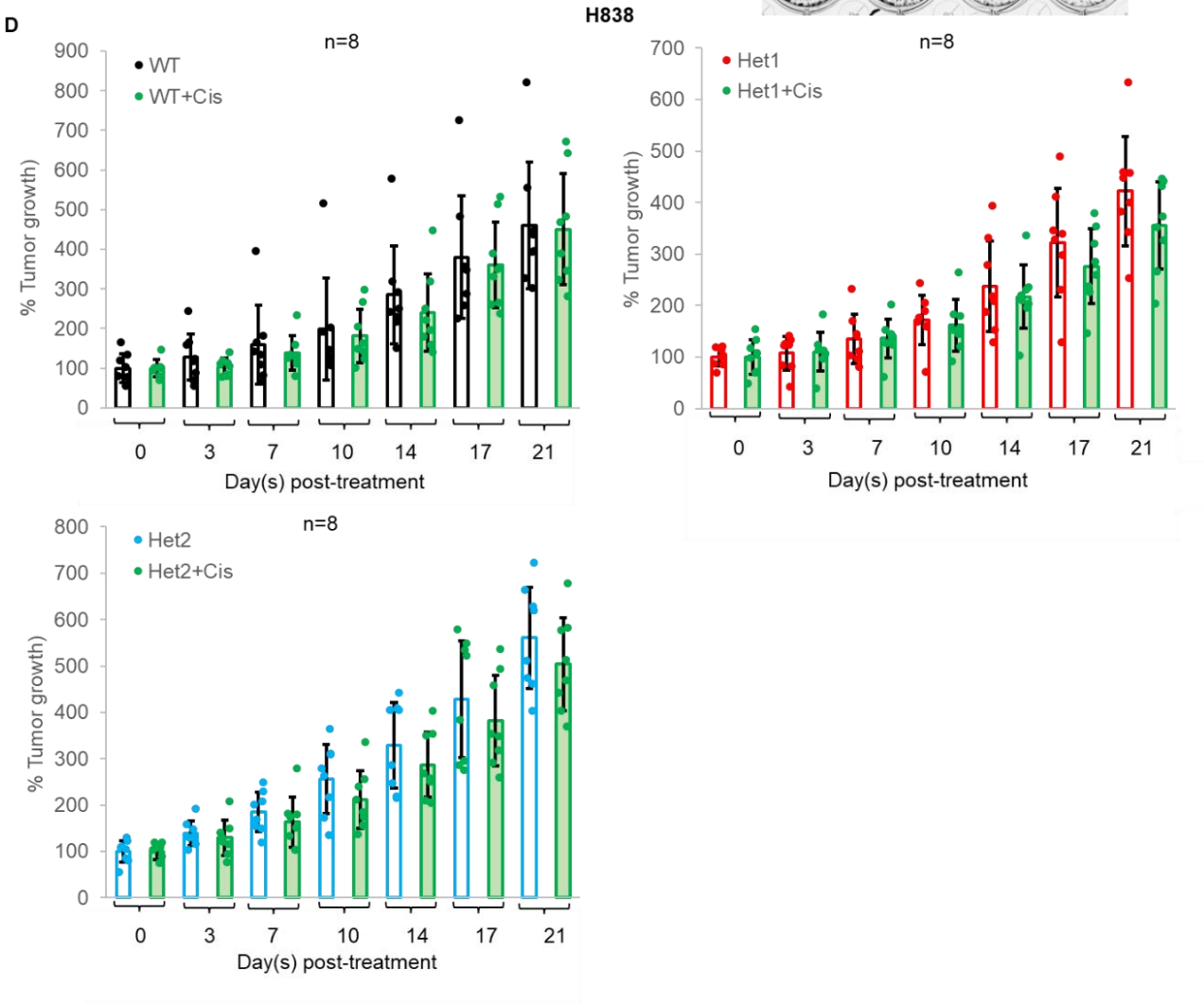

C
H358
WT
Het
200 μM


D
H358
Cell survival (%)
120
100
80
60
40
20
0
WT
Het
*
**
***
Het to WT
*p<0.05
**p<0.005
***p<0.00001
0
0.25
0.5
1
Cis (μM)
WT
Het

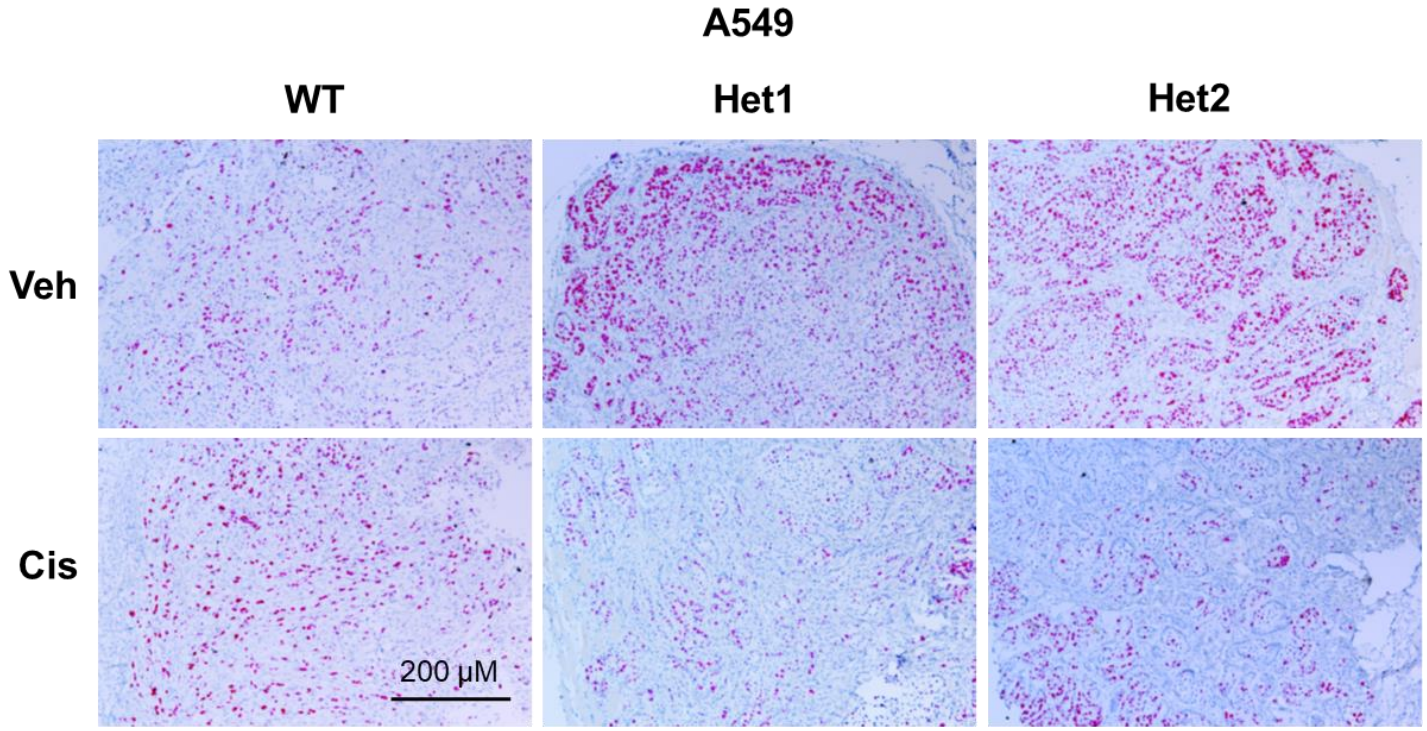

E
H358
% Tumor growth
600
500
400
300
200
100
0
WT (n=8)
WT+Cis (n=6)
0
3
7
10
14
17
21
Day(s) post-treatment
% Tumor growth
900
800
700
600
500
400
300
200
100
0
Het (n=10)
Het+Cis (n=9)
*
*
*
**
0
3
7
10
14
17
21
Day(s) post-treatment

Supplemental Figure S11: Comparison of CERK expression, AKT phosphorylation, ferroptosis induction, cell survival and cisplatin responsiveness level in CERK Het versus WT H838 cells. CERK Het or WT H838 cells (LUAD) were grown to reach approximately 40-50% confluency. (A) RT-qPCR for CERK were undertaken from RNAs samples prepared from cell lysates. (B) Cell lysates were subjected to Western immunoblotting. (C) Cells were treated with cisplatin for 24 hrs before clonogenic survival assay were undertaken. (D) H838 cells with CERK WT (+/+) or Het (+/-) were injected into the flanks of nude mice (10^6 cells/mice). Mice developing tumors with approximate 100 mm^3 volume were treated with cisplatin (3mmg/kg body weight) twice/week for 3 weeks. Tumor size (volume) was measured twice/week. Data in (A) and (D) are means ± SD; data in (C) are mean; n = 4 in (A) and n=8 in (C) from two independent occasions; n=8 in (D). n = 1 tumor. Unless otherwise noted by an * or depicted p-value, data are not significant between depicted groups; $p > 0.05$.

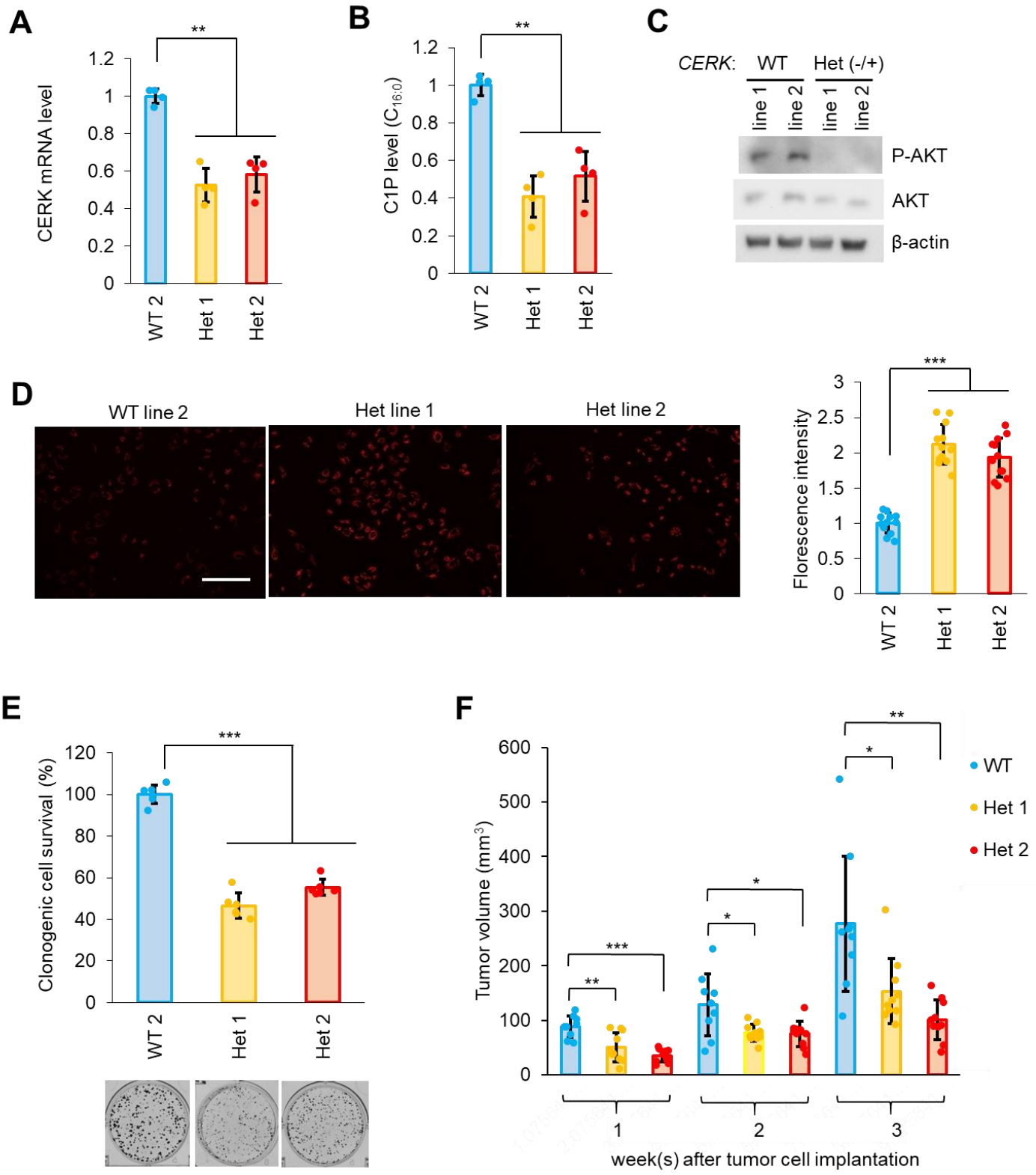

A
H838
***
CERK mRNA level
1.2
1
0.8
0.6
0.4
0.2
0
WT
Het 1
Het 2
B
H838
WT
Het 1
Het 2
P-AKT
AKT
TFR1
β-actin
C
H838
Cell survival (%)
120
100
80
60
40
20
0
0
0.25
0.5
1
Cis (μM)
WT
Het 1
Het 2
WT
Het 1
Het 2


D
H838
n=8
WT
WT+Cis
% Tumor growth)
900
800
700
600
500
400
300
200
100
0
0
3
7
10
14
17
21
Day(s) post-treatment
n=8
Het1
Het1+Cis
% Tumor growth)
700
600
500
400
300
200
100
0
0
3
7
10
14
17
21
Day(s) post-treatment
n=8
Het2
Het2+Cis
% Tumor growth)
800
700
600
500
400
300
200
100
0
0
3
7
10
14
17
21
Day(s) post-treatment

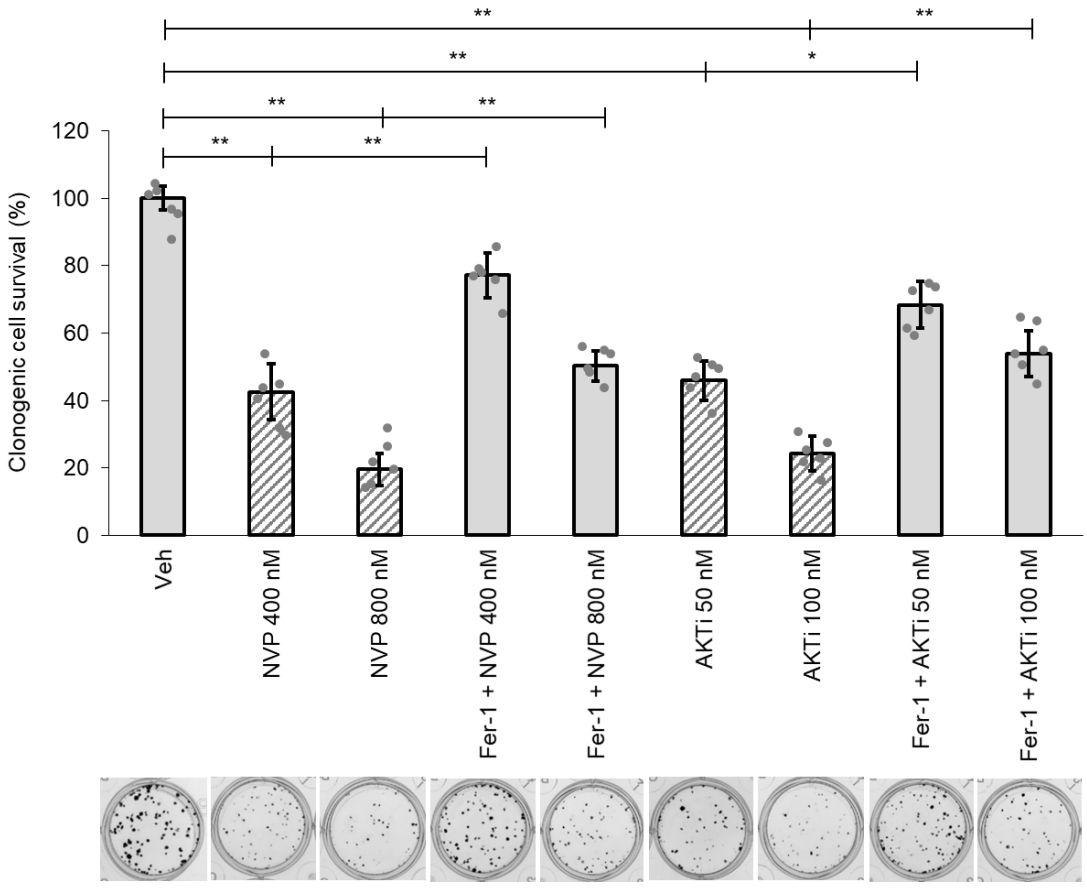
Supplemental Figure S12: Tumors formed by *KRAS*-mutant NSCLC cells with Het (+/-) *CERK* are more sensitive to cisplatin treatment regarding cell proliferation compared to WT *CERK*. Ki67 immunohistochemical analysis was performed with tumor sections in Figure 5D (A549 cells; LUAD). Sections were also counterstained with hematoxylin to visualize cell nucleus and tissue architecture. Scale bar = 200 µm.



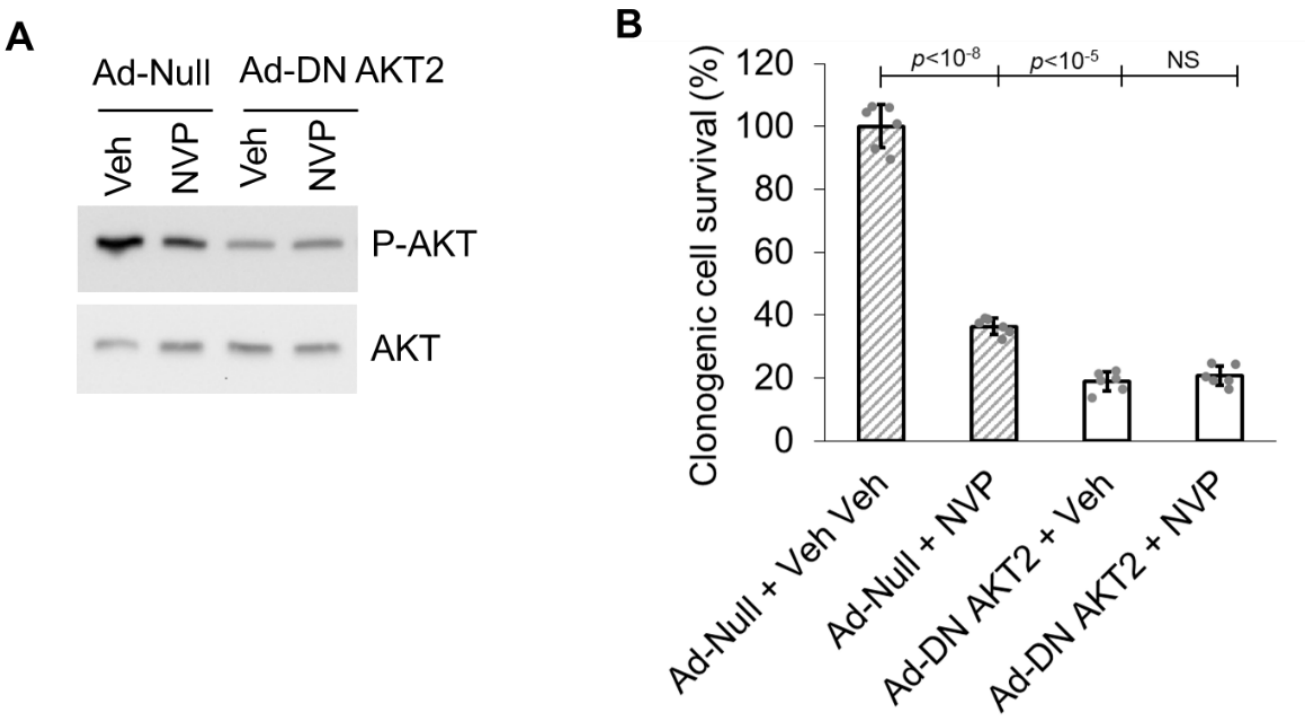
Supplemental Figure S13: Characterization of the *CERK*$^{+/-}$ A549 cells. *CERK*$^{+/-}$ (Het) or *CERK*$^{+/+}$ (WT) A549 cells (LUAD) were grown to reach approximately 40-50% confluency. (**A**) RT-qPCR for CERK was undertaken from RNA samples prepared from cell lysates. (**B, C**) Cell lysates were subjected to mass spectrometry analysis for C1P ($C_{16:0}$) levels (B) or Western immunoblotting (C). (**D, E**) Cells were subjected to MMP (D) or clonogenic survival (E) assay. (**F**) A549 cells with WT or Het *CERK* were injected into the flanks of nude mice. Tumor size was measured every week for 3 weeks (n = 1 tumor for these data). Data in graphs are means ± SD; n = 4-6 from two independent occasions in (A-E); n=9-10 in (F). Scale bar = 200 μm; *p<0.05, **p<0.005, ***p<0.0005. Unless otherwise noted by an * or depicted p-value, data are not significant between depicted groups; p > 0.05.


A
CERK mRNA level
WT 2
Het 1
Het 2
B
C1P level (C16:0)
C
CERK: WT Het (-/+)
line 1
line 2
P-AKT
AKT
β-actin
D
WT line 2
Het line 1
Het line 2
Florescence intensity
E
Clonogenic cell survival (%)
F
Tumor volume (mm3)
WT
Het 1
Het 2
week(s) after tumor cell implantation

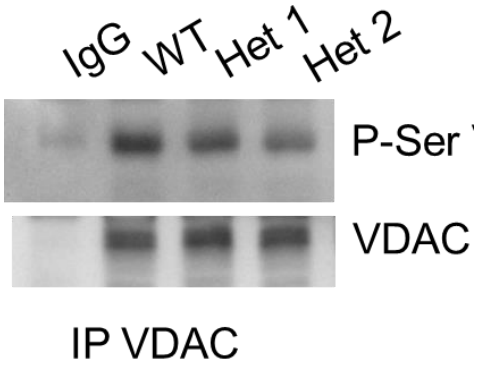
Supplemental Figure S14: AKT inhibition induces ferroptotic cell death. A549 cells were treated with vehicle (Veh) or ferroptosis inhibitor [Ferrostatin-1 (Fer-1), 5 uM] for 1 hour followed by 16-hour treatment with Veh, NVP-231 (400-800 nM) or AKT inhibitor, BEZ235 (AKTi; 50-100 nM). Cells were then subjected to clonogenic survival assays. Data are means ± SD; n = 6 from two independent occasions. *$p<10^{-4}$, **$p<10^{-7}$. Unless otherwise noted by an * or depicted p-value, data are not significant between depicted groups; $p > 0.05$.



Supplemental Figure S15: Suppression of AKT pathway blocks the effect of CERK inhibition on AKT activation and cell survival in Mut *KRAS* NSCLC cells. A549 cells (LUAD) were transfected with adenovirus control or expressing dominant negative AKT2 for 48 hours followed by 6-hr (A) or 24-hr (B) treatment of NVP 231 (400 nM). Cells were then subjected to SDS-PAGE/immunoblotting (A) or clonogenic cell survival assay (B). Data in (B) are means ± SD; n = 6 from two independent occasions. Unless otherwise noted by an * or depicted p-value, data are not significant between depicted groups; p > 0.05 (NS).



Supplemental Figure S16: CERK Het (+/-) cells have less phospho-serine VDAC. Mitochondria fraction from WT/Het1/Het2 CERK A549 cells (LUAD) were subjected to IP assay for VDAC1 followed by SDS-PAGE/immunoblotting.



Supplemental Figure S17: Re-expression of CERK in WT versus Het CERK cells. Het $^{(+/-)}$ or WT $^{(+/+)}$ *CERK* cells (A549 (LUAD)) were transfected with control (Con) or V5-tagged CERK (V5-CE) plasmid for 24 hours. Then cells were lysed for Western immunoblotting (A) or were subjected to clonogenic cell survival assay (B). Data in graphs are means ± SD; n = 6 from two independent occasions. *$p<10^{-4}$, **$p<10^{-7}$. Unless otherwise noted by an * or depicted p-value, data are not significant between depicted groups; $p > 0.05$.