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Supplemental Information

Low-Dose Vertical Inhibition of the RAF-MEK-ERK Cascade Causes Apoptotic Death of KRAS Mutant Cancers

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Figure S1

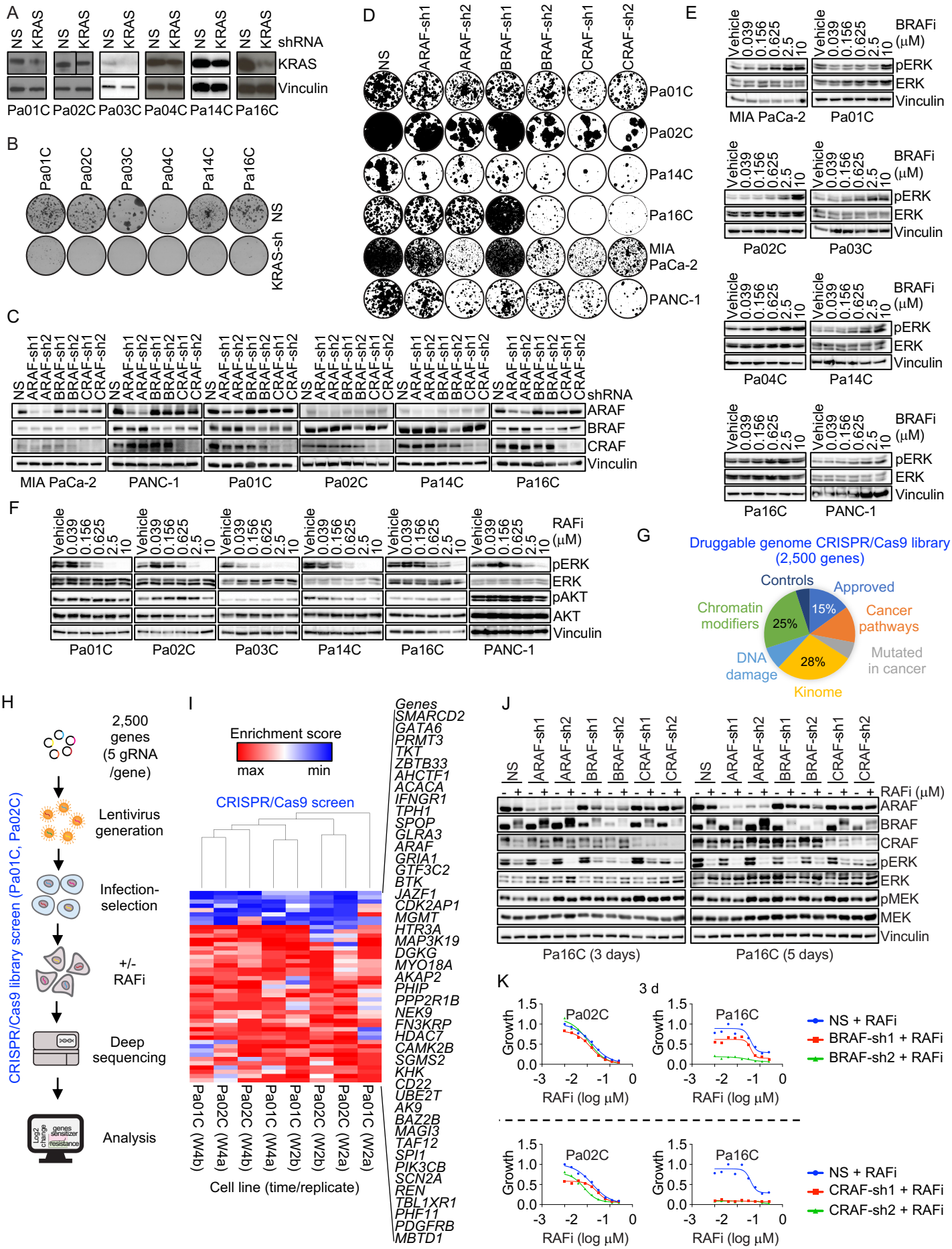
A
NS
KRAS
shRNA
KRAS
Vinculin
Pa01C Pa02C Pa03C Pa04C Pa14C Pa16C
B
Pa01C
Pa02C
Pa03C
Pa04C
Pa14C
Pa16C
NS
KRAS-sh
C
ARAF-sh1
ARAF-sh2
BRAF-sh1
BRAF-sh2
CRAF-sh1
CRAF-sh2
shRNA
ARAF
BRAF
CRAF
Vinculin
MIA PaCa-2
PANC-1
Pa01C
Pa02C
Pa14C
Pa16C
D
NS
ARAF-sh1
ARAF-sh2
BRAF-sh1
BRAF-sh2
CRAF-sh1
CRAF-sh2
Pa01C
Pa02C
Pa14C
Pa16C
MIA PaCa-2
PANC-1
E
Vehicle
0.039
0.156
0.625
2.5
10
BRAFi (μM)
pERK
ERK
Vinculin
MIA PaCa-2
Pa01C
Pa02C
Pa03C
Pa04C
Pa14C
Pa16C
PANC-1
F
RAFi (μM)
pERK
ERK
pAKT
AKT
Vinculin
Pa01C
Pa02C
Pa03C
Pa14C
Pa16C
PANC-1
G
Druggable genome CRISPR/Cas9 library (2,500 genes)
Controls
Approved
Chromatin modifiers
25%
15%
Cancer pathways
DNA damage
28%
Mutated in cancer
Kinome
H
CRISPR/Cas9 library screen (Pa01C, Pa02C)
2,500 genes (5 gRNA /gene)
Lentivirus generation
Infection-selection
+/- RAFi
Deep sequencing
Log2 change
genes
sensitizer
resistance
Analysis
I
Enrichment score
max
min
CRISPR/Cas9 screen
Genes
SMARCD2
GATA6
PRMT3
TKT
ZBTB33
AHCTF1
ACACA
IFNGR1
TPH1
SPOP
GLRA3
ARAF
GRIA1
GTF3C2
BTK
JAZF1
CDK2AP1
MGMT
HTR3A
MAP3K19
DGKG
MYO18A
AKAP2
PHIP
PPP2R1B
NEK9
FN3KRP
HDAC7
CAMK2B
SGMS2
KHK
CD22
UBE2T
AK9
BAZ2B
MAGI3
TAF12
SPI1
PIK3CB
SCN2A
REN
TBL1XR1
PHF11
PDGFRB
MBTD1
Pa01C (W4b)
Pa02C (W4a)
Pa02C (W4b)
Pa01C (W4a)
Pa01C (W2b)
Pa02C (W2b)
Pa02C (W2a)
Pa01C (W2a)
Cell line (time/replicate)
J
NS
ARAF-sh1
ARAF-sh2
BRAF-sh1
BRAF-sh2
CRAF-sh1
CRAF-sh2
RAFi (μM)
ARAF
BRAF
CRAF
pERK
ERK
pMEK
MEK
Vinculin
Pa16C (3 days)
Pa16C (5 days)
K
3 d
Pa02C
Pa16C
Growth
RAFi (log μM)
NS + RAFi
BRAF-sh1 + RAFi
BRAF-sh2 + RAFi
CRAF-sh1 + RAFi
CRAF-sh2 + RAFi

Figure S1. All RAF Isoforms are Necessary for PDAC Growth, Related to Figure 1

(A) *KRAS* mutant PDAC cell lines were infected by lentivirus vectors encoding nonspecific (NS) control or *KRAS* shRNA (72 hr). Cell lysates were immunoblotted to determine levels of KRAS and vinculin (control for total protein).

(B) PDAC cell lines were infected by lentivirus vectors encoding NS control or *KRAS* shRNAs (72 hr) and colonies were stained with crystal violet ~10 days after plating. Representative images are shown.

(C) PDAC cell lines were infected by lentivirus vectors encoding NS control or two shRNAs targeting *ARAF*, *BRAF* or *CRAF* (72 hr). Cell lysates were immunoblotted to determine levels of ARAF, BRAF, CRAF and vinculin.

(D) PDAC cell lines were infected by lentivirus vectors encoding NS control, *ARAF*, *BRAF* or *CRAF* shRNAs (72 hr). Colonies were stained by crystal violet ~10 days after plating. Representative images are shown.

(E) PDAC cell lines were treated with mutant BRAF-specific inhibitor vemurafenib (BRAFi, 0.04-10 μM, 72 hr). Cell lysates were immunoblotted to determine levels of phosphorylated ERK, total ERK and vinculin.

(F) PDAC cell lines were treated with the pan-RAF inhibitor LY3009120 (RAFi, 0.04-10 μM, 72 hr). Cell lysates were immunoblotted to determine levels of phosphorylated ERK, total ERK, phosphorylated AKT, total AKT and vinculin.

(G) The composition of the loss-of-function CRISPR/Cas9 druggable genome (2,500 genes) library screen.

(H) CRISPR/Cas9 druggable genome library screen schematic.

(I) CRISPR screen. PDAC cell lines were infected with a pooled CRISPR library and treated with vehicle control or RAFi (w2: 2 weeks, w4: 4 weeks; a and b indicate replicate samples). The enrichment score indicates either enrichment (red) or depletion (blue) of barcoded sequences corresponding to the indicated genes in cells treated with RAFi relative to vehicle control.

(J) Pa16C cells were infected by lentivirus vectors encoding NS control or two distinct shRNAs for *ARAF*, *BRAF* or *CRAF* for 72 hr, and treated with LY3009120 (RAFi, 0.3 μM) for additional 72 or 120 hr (related to Figure 1E). Cell lysates were immunoblotted to determine levels of ARAF, BRAF, CRAF, phosphorylated ERK, total ERK, phosphorylated MEK (Ser 298), total MEK and vinculin.

(K) Related to Figure 1E. Pa02C and Pa16C cell lines were infected by lentivirus vectors encoding NS or two distinct *BRAF* or *CRAF* shRNAs (72 hr) and treated with LY3009120 (RAFi, 0.01-2.5 μM) for additional 120 hr. Proliferation was measured by Calcein AM cell viability assay. Data are the mean average of three technical replicates. Error bars are shown as ± S.E.M.

Figure S2

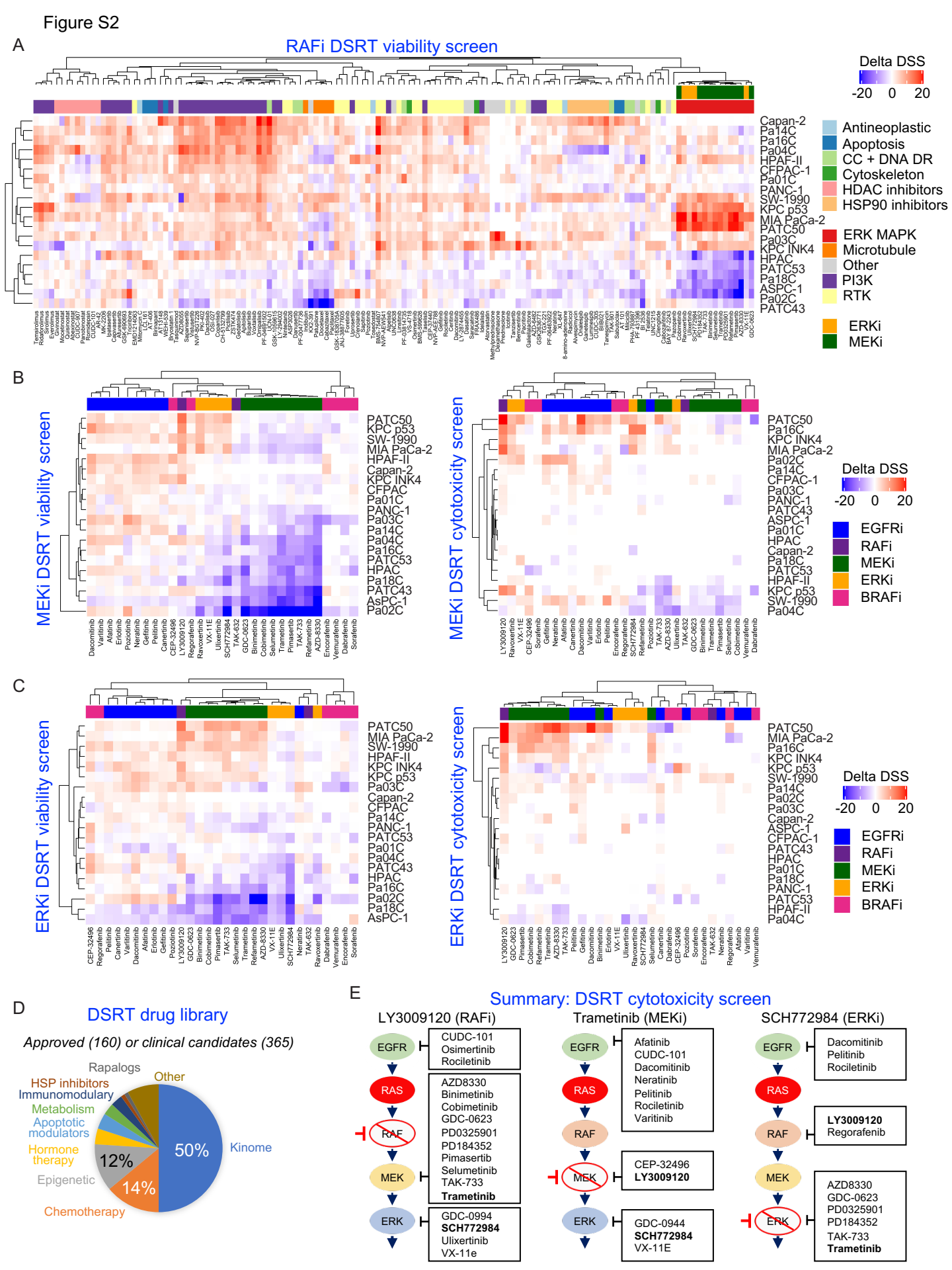
A
RAFi DSRT viability screen
Delta DSS
-20 0 20
Capan-2
Pa14C
Pa16C
Pa04C
HPAF-II
CFPAC-1
Pa01C
PANC-1
SW-1990
KPC p53
MIA PaCa-2
PATC50
Pa03C
KPC INK4
HPAC
PATC53
Pa18C
ASPC-1
Pa02C
PATC43
Antineoplastic
Apoptosis
CC + DNA DR
Cytoskeleton
HDAC inhibitors
HSP90 inhibitors
ERK MAPK
Microtubule
Other
PI3K
RTK
ERKi
MEKi
B
MEKi DSRT viability screen
PATC50
KPC p53
SW-1990
MIA PaCa-2
HPAF-II
Capan-2
KPC INK4
CFPAC
Pa01C
PANC-1
Pa03C
Pa14C
Pa04C
Pa16C
PATC53
HPAC
Pa18C
PATC43
AsPC-1
Pa02C
MEKi DSRT cytotoxicity screen
PATC50
Pa16C
KPC INK4
MIA PaCa-2
Pa02C
Pa14C
CFPAC-1
Pa03C
PANC-1
PATC43
ASPC-1
Pa01C
HPAC
Capan-2
Pa18C
PATC53
HPAF-II
KPC p53
SW-1990
Pa04C
Delta DSS
-20 0 20
EGFRi
RAFi
MEKi
ERKi
BRAFi
C
ERKi DSRT viability screen
PATC50
MIA PaCa-2
SW-1990
HPAF-II
KPC INK4
KPC p53
Pa03C
Capan-2
CFPAC
Pa14C
PANC-1
PATC53
Pa01C
Pa04C
PATC43
HPAC
Pa16C
Pa02C
Pa18C
AsPC-1
ERKi DSRT cytotoxicity screen
PATC50
MIA PaCa-2
Pa16C
KPC INK4
KPC p53
SW-1990
Pa14C
Pa02C
Pa03C
Capan-2
ASPC-1
CFPAC-1
PATC43
HPAC
Pa01C
Pa18C
PANC-1
PATC53
HPAF-II
Pa04C
Delta DSS
-20 0 20
EGFRi
RAFi
MEKi
ERKi
BRAFi
D
DSRT drug library
Approved (160) or clinical candidates (365)
Rapalogs
Other
HSP inhibitors
Immunomodulary
Metabolism
Apoptotic modulators
Hormone therapy
Epigenetic
Chemotherapy
50%
Kinome
12%
14%
E
Summary: DSRT cytotoxicity screen
LY3009120 (RAFi)
EGFR
CUDC-101
Osimertinib
Rociletinib
RAS
AZD8330
Binimetinib
Cobimetinib
GDC-0623
PD0325901
PD184352
Pimasertib
Selumetinib
TAK-733
Trametinib
RAF
MEK
ERK
GDC-0994
SCH772984
Ulixertinib
VX-11e
Trametinib (MEKi)
EGFR
Afatinib
CUDC-101
Dacomitinib
Neratinib
Pelitinib
Rociletinib
Varitinib
RAS
RAF
MEK
CEP-32496
LY3009120
ERK
GDC-0944
SCH772984
VX-11E
SCH772984 (ERKi)
EGFR
Dacomitinib
Pelitinib
Rociletinib
RAS
RAF
LY3009120
Regorafenib
MEK
ERK
AZD8330
GDC-0623
PD0325901
PD184352
TAK-733
Trametinib

Figure S2. Drug Sensitivity Resistance Testing Screen (DSRT), Related to Figure 2

A) Human or mouse *KRAS* mutant PDAC cell lines were treated with a 525-inhibitor library with or without LY3009120 (RAFi, 2 μM, 72 hr). Proliferation was measured by Calcein AM viability assay. Drug sensitivity score (δDSS) was used to quantify inhibitor responses. Red (> additive), blue (< additive) or white (no effect).

(B) Human or mouse *KRAS* mutant PDAC cell lines were treated with a 525-inhibitor library with or without trametinib (MEKi, 0.025 μM, 72 hr). Proliferation was measured by Calcein AM viability assay (left), and cell death was measured by CellTox Green cytotoxicity assay (right). Inhibitor responses were plotted as δDSS. Red (> additive), blue (< additive) or white (no effect).

(C) Human or mouse *KRAS* mutant PDAC cell lines were treated as described in Figure S2B with SCH772984 (ERKi, 0.1 μM, 72 hr). Proliferation and cell death measured as described in Figure S2B.

(D) The composition of the DSRT screen.

(E) Top hits summarized for the RAFi, MEKi or ERKi DSRT viability and cytotoxicity screens.

Figure S3

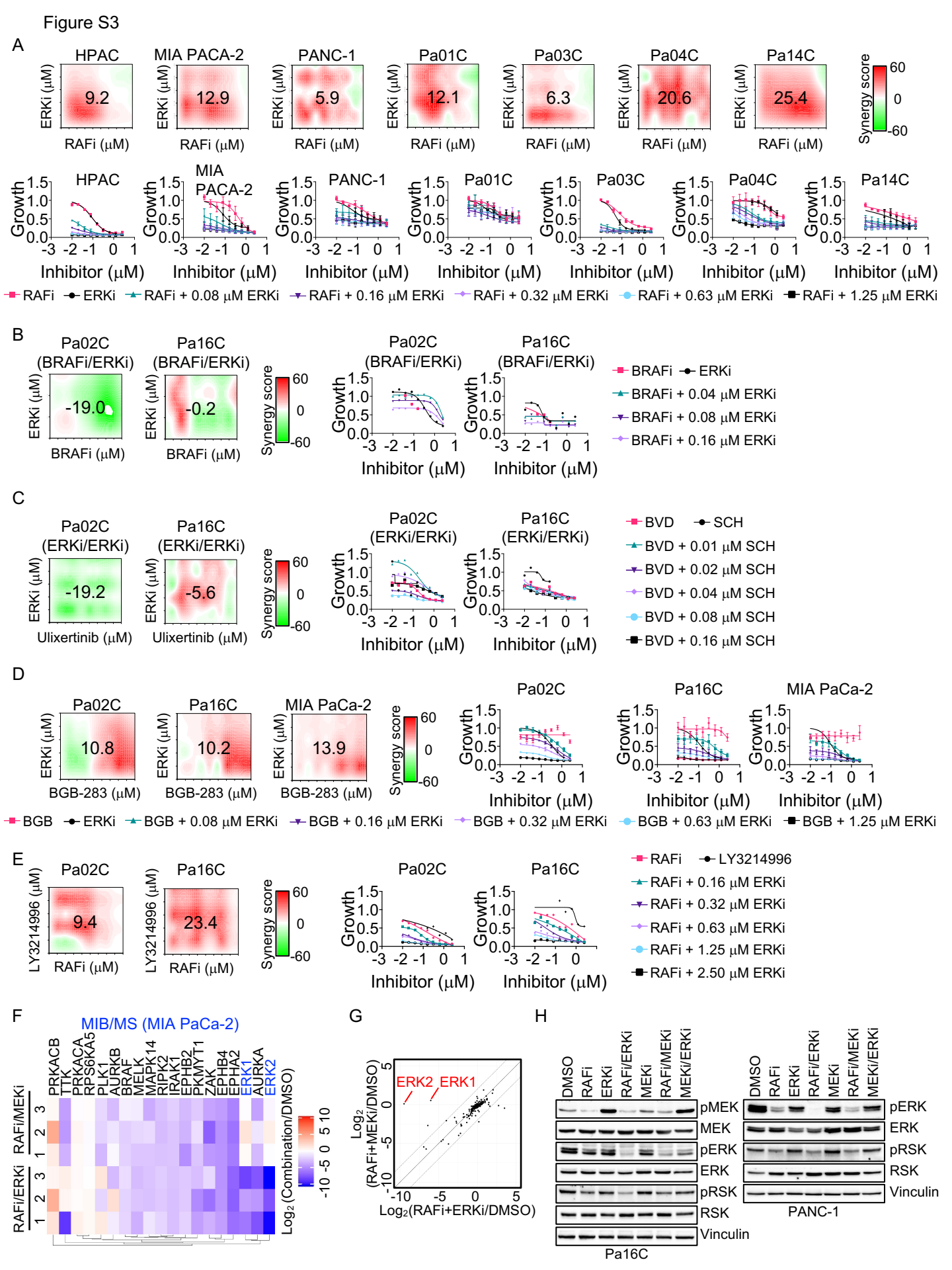
A
HPAC
MIA PACA-2
PANC-1
Pa01C
Pa03C
Pa04C
Pa14C
9.2
12.9
5.9
12.1
6.3
20.6
25.4
ERKi (μM)
RAFi (μM)
Synergy score
60
0
-60
Growth
Inhibitor (μM)
RAFi
ERKi
RAFi + 0.08 μM ERKi
RAFi + 0.16 μM ERKi
RAFi + 0.32 μM ERKi
RAFi + 0.63 μM ERKi
RAFi + 1.25 μM ERKi
B
Pa02C (BRAFi/ERKi)
Pa16C (BRAFi/ERKi)
-19.0
-0.2
BRAFi (μM)
BRAFi
ERKi
BRAFi + 0.04 μM ERKi
BRAFi + 0.08 μM ERKi
BRAFi + 0.16 μM ERKi
C
Pa02C (ERKi/ERKi)
Pa16C (ERKi/ERKi)
-19.2
-5.6
Ulixertinib (μM)
BVD
SCH
BVD + 0.01 μM SCH
BVD + 0.02 μM SCH
BVD + 0.04 μM SCH
BVD + 0.08 μM SCH
BVD + 0.16 μM SCH
D
Pa02C
Pa16C
MIA PaCa-2
10.8
10.2
13.9
BGB-283 (μM)
BGB
ERKi
BGB + 0.08 μM ERKi
BGB + 0.16 μM ERKi
BGB + 0.32 μM ERKi
BGB + 0.63 μM ERKi
BGB + 1.25 μM ERKi
E
Pa02C
Pa16C
9.4
23.4
LY3214996 (μM)
RAFi (μM)
RAFi
LY3214996
RAFi + 0.16 μM ERKi
RAFi + 0.32 μM ERKi
RAFi + 0.63 μM ERKi
RAFi + 1.25 μM ERKi
RAFi + 2.50 μM ERKi
F
MIB/MS (MIA PaCa-2)
PRKACB
TTK
PRKACA
RPS6KA5
PLK1
AURKB
BRAF
MELK
MAPK14
RIPK2
IRAK1
EPHB2
PKMYT1
ZAK
EPHB4
EPHA2
ERK1
AURKA
ERK2
RAFi/MEKi
RAFi/ERKi
Log2 (Combination/DMSO)
G
ERK2
ERK1
Log2 (RAFi+MEKi/DMSO)
Log2(RAFi+ERKi/DMSO)
H
DMSO
RAFi
ERKi
RAFi/ERKi
MEKi
RAFi/MEKi
MEKi/ERKi
pMEK
MEK
pERK
ERK
pRSK
RSK
Vinculin
Pa16C
PANC-1

Figure S3. Concurrent RAF and ERK Inhibition Causes Synergistic Growth Suppression, Related to Figure 2

(A) Indicated *KRAS* mutant PDAC cell lines were treated with the vehicle control (lower left of each graph), LY3009120 (RAFi, 0.01-2.5 μM, 1:2 dilution) and SCH772984 (ERKi, 0.08-1.25 μM, 1:2 dilution) alone or in combination for 120 hr. Proliferation was measured by Calcein AM cell viability assay. Representative bliss synergy score heatmaps of three biological replicates (up). Red (synergy), green (antagonism), white (no effect). Averaged synergy scores presented on the synergy heatmaps. Averaged dose response curves of three biological replicates (down). Error bars are shown as ±S.E.M.

(B) PDAC cell lines Pa02C and Pa16C were treated with the vehicle control (lower left of each graph), vemurafenib (BRAFi, 0.01-2.5 μM, 1:2 dilution) and SCH772984 (ERKi, 0.08-1.25 μM, 1:2 dilution) alone or in combination for 120 hr. Bliss synergy scores and dose response curves are calculated as in Figure S2F.

(C) Pa02C and Pa16C were treated with the vehicle control (lower left of each graph), the ERK1/2 inhibitor ulixertinib (BVD-523, 0.01-2.5 μM, 1:2 dilution) and SCH772984 (ERKi, 0.08-1.25 μM, 1:2 dilution) alone or in combination for 120 hr. Bliss synergy scores and dose response curves are calculated as in Figure S2F.

(D) Pa02C, Pa16C and MIA PaCa-2 cell lines were treated with the vehicle control (lower left of each graph), the pan-RAF inhibitor lifirafenib/BGB-283 (0.01-2.5 μM, 1:2 dilution) and SCH772984 (ERKi, 0.08-1.25 μM,1:2 dilution) alone or in combination for 120 hr. Bliss synergy scores and dose response curves are calculated as in Figure S2F.

(E) *KRAS* mutant PDAC cell lines Pa02C and Pa16C were treated with the vehicle control (lower left of each graph), LY3009120 (RAFi, 0.01-2.5 μM, 1:2 dilution) and the ERK1/2 inhibitor LY3214996 (0.08-1.25 μM, 1:2 dilution) for 120 hr. Bliss synergy scores and dose response curves are shown as in Figure S2F.

(F) Kinome-wide chemical proteomics screen by multiplexed kinase inhibitor beads and mass spectrometry (MIB/MS). MIA PaCa-2 cells were treated with the vehicle control DMSO, LY3009120 (RAFi, 0.3 μM), trametinib (MEKi, 0.5 nM), SCH772984 (ERKi, 0.04 μM) alone or in combination for 72 hr. Fold changes of significantly altered kinases (p value ≤ 0.05) are plotted. Each treatment is normalized to its respective vehicle control.

(G) Average $\log_2$ fold-change values (over vehicle control) for each kinase identified in MIB/MS were plotted along the x-axis (RAFi+ERKi) or y-axis (RAFi+MEKi). The dashed lines indicate differences in $\log_2$ fold-change values of 1 or -1.

(H) Pa16C and PANC-1 cells were treated with LY3009120 (RAFi, 0.3 μM, trametinib (MEKi, 0.5 nM) or SCH772984 (ERKi, 0.04 μM) alone or in combination as indicated (120 hr). Cell lysates were immunoblotted to determine the levels of phosphorylated and total MEK, ERK, RSK proteins and the loading control vinculin.

Figure S4

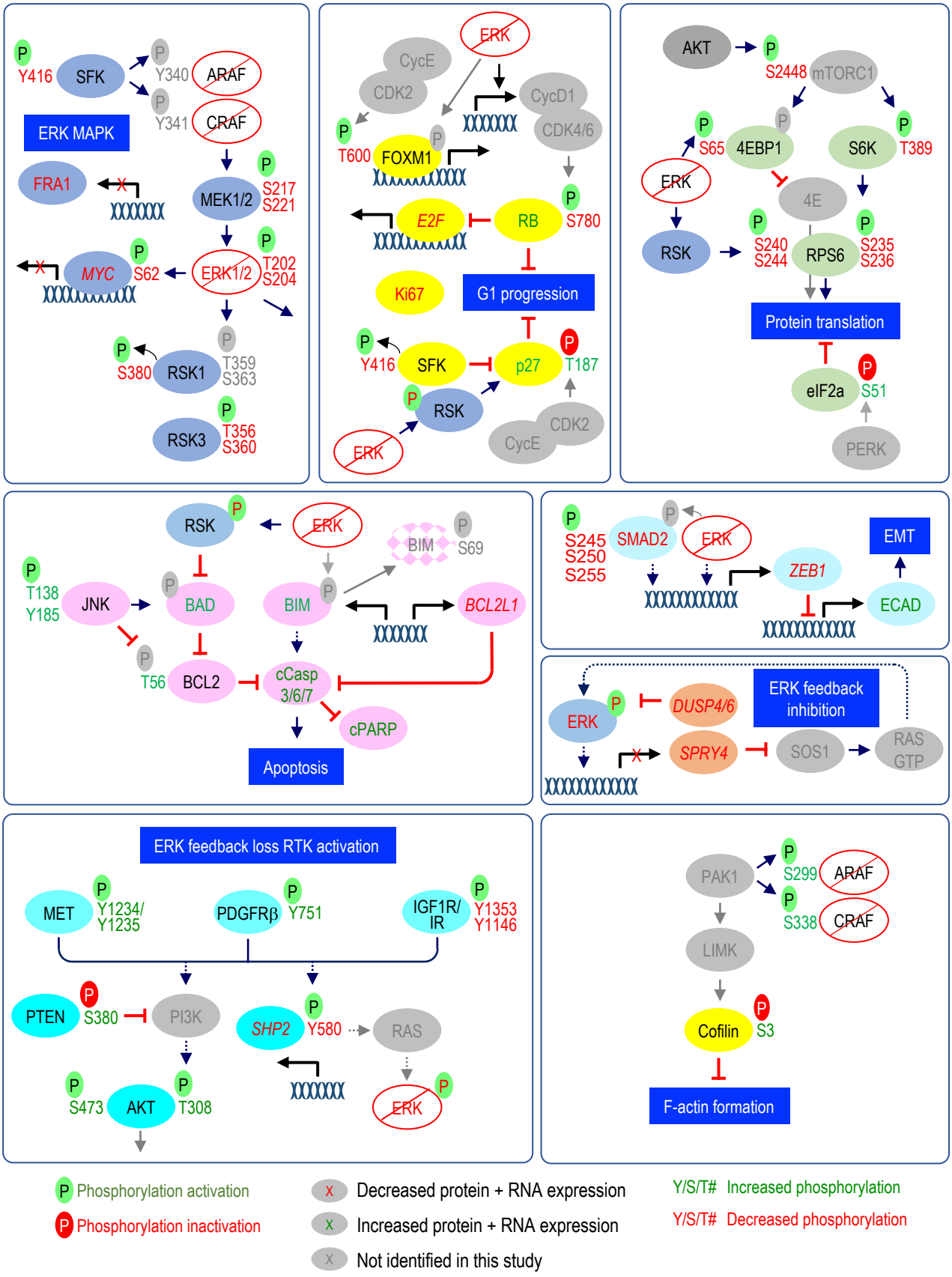

Figure S4. The pathways altered by the concurrent RAF and ERK inhibition, Related to Figure 3

The processes altered by LY3009120 (RAFi, 0.3 μM) and SCH772984 (ERKi, 0.04 μM) combination treatment revealed by reverse phase protein array (RPPA) and RNA sequencing.

Figure S5

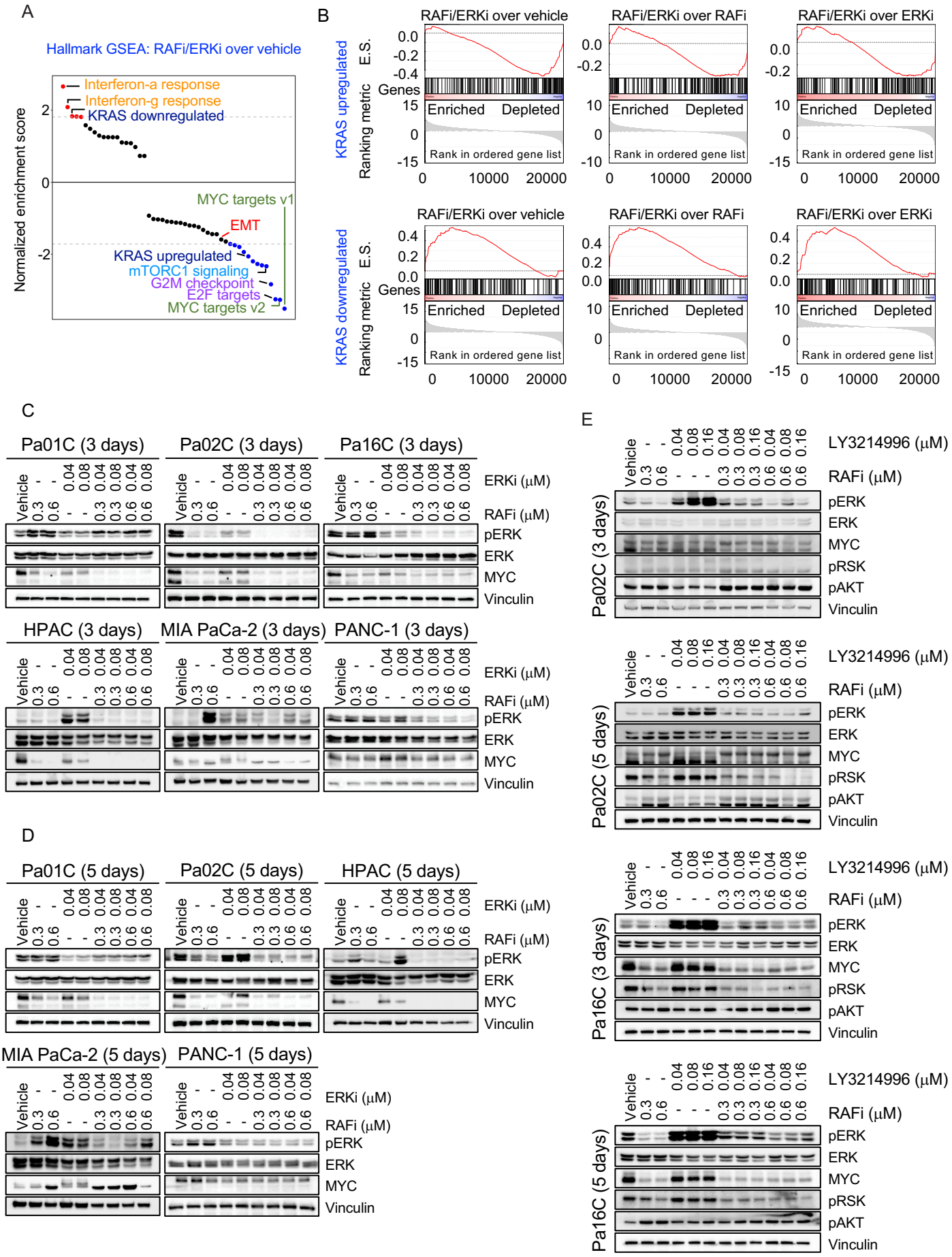
A
Hallmark GSEA: RAFi/ERKi over vehicle
Normalized enrichment score
Interferon-a response
Interferon-g response
KRAS downregulated
MYC targets v1
EMT
KRAS upregulated
mTORC1 signaling
G2M checkpoint
E2F targets
MYC targets v2
B
RAFi/ERKi over vehicle
RAFi/ERKi over RAFi
RAFi/ERKi over ERKi
KRAS upregulated
KRAS downregulated
E.S.
Genes
Ranking metric
Enriched
Depleted
Rank in ordered gene list
C
Pa01C (3 days)
Pa02C (3 days)
Pa16C (3 days)
HPAC (3 days)
MIA PaCa-2 (3 days)
PANC-1 (3 days)
ERKi (μM)
RAFi (μM)
pERK
ERK
MYC
Vinculin
D
Pa01C (5 days)
Pa02C (5 days)
HPAC (5 days)
MIA PaCa-2 (5 days)
PANC-1 (5 days)
E
LY3214996 (μM)
RAFi (μM)
Pa02C (3 days)
Pa02C (5 days)
Pa16C (3 days)
Pa16C (5 days)
pERK
ERK
MYC
pRSK
pAKT
Vinculin

Figure S5. Concurrent RAF and ERK inhibition Blocks ERK Reactivation, Related to Figure 3

(A) Gene set enrichment analysis (GSEA) of RNA transcripts of MIA PaCa-2, Pa02C, Pa14C and Pa16C cell lines. Enriched or depleted gene sets treated with RAFi + ERKi combination compared to the vehicle control DMSO are shown (24 hr).

(B) Gene set enrichment analysis (GSEA) of hallmark KRAS signaling. Altered gene expressions are plotted as enrichment scores (E.S.). PDAC cells were treated with LY3009120 (RAFi, 0.3 μM) and SCH772984 (ERKi, 0.04 μM) for 24 hr and normalized to the vehicle control DMSO (left), RAFi (middle), or ERKi (right). Data shown are the average of four PDAC cell lines MIA PaCa-2, Pa02C, Pa14C and Pa16C.

(C) Indicated *KRAS* mutant PDAC cell lines were treated with the vehicle control DMSO, LY3009120 (RAFi, 0.3 or 0.6 μM), SCH772984 (ERKi, 0.04 or 0.08 μM) alone or in combination for 72 hr or

(D) 120 hr. Cell lysates were immunoblotted to determine levels of phosphorylated ERK, total ERK, MYC and vinculin.

(E) Pa02C and Pa16C cells were treated with the vehicle control DMSO, LY3009120 (RAFi, 0.3 or 0.6 μM), the ERK1/2 inhibitor LY3214996 (0.04, 0.08 or 0.16 μM) alone or in combination for 72 or 120 hr. Cell lysates were immunoblotted to determine levels of phosphorylated and total ERK, MYC, phosphorylated RSK, phosphorylated AKT and vinculin.

Figure S6

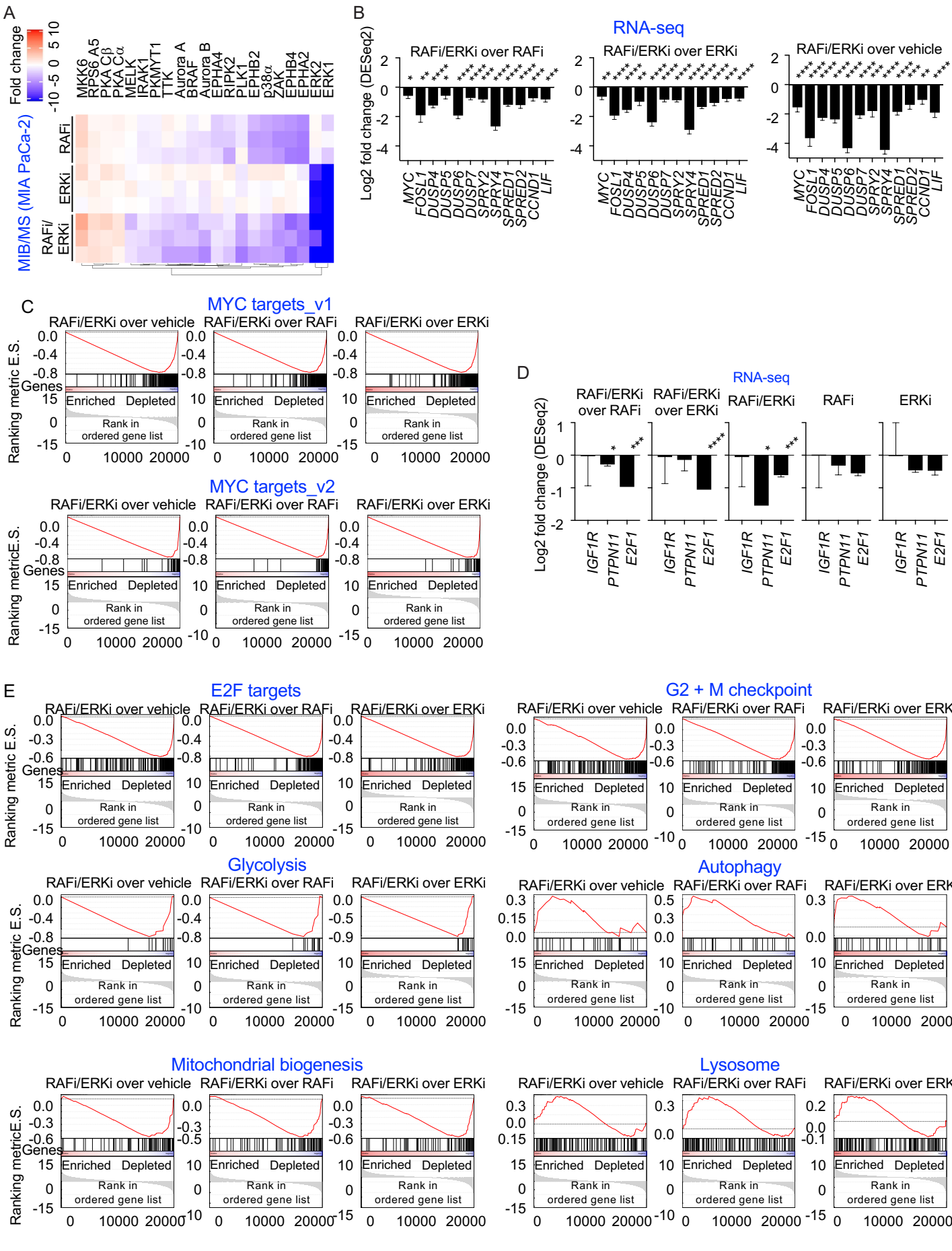
A
Fold change
MIB/MS (MIA PaCa-2)
RAFi
ERKi
RAFi/ERKi
MKK6
RPS6 A5
PKA Cβ
PKA Cα
MELK
IRAK1
PKMYT1
TTK
Aurora A
BRAF
Aurora B
EPHA4
RIPK2
PLK1
EPHB2
p38α
ZAK
EPHB4
EPHA2
ERK2
ERK1
B
RNA-seq
RAFi/ERKi over RAFi
RAFi/ERKi over ERKi
RAFi/ERKi over vehicle
Log2 fold change (DESeq2)
MYC
FOSL1
DUSP4
DUSP5
DUSP6
DUSP7
SPRY2
SPRY4
SPRED1
SPRED2
CCND1
LIF
C
MYC targets_v1
MYC targets_v2
RAFi/ERKi over vehicle
RAFi/ERKi over RAFi
RAFi/ERKi over ERKi
Ranking metric E.S.
Genes
Enriched
Depleted
Rank in ordered gene list
D
RNA-seq
RAFi/ERKi over RAFi
RAFi/ERKi over ERKi
RAFi/ERKi
RAFi
ERKi
Log2 fold change (DESeq2)
IGF1R
PTPN11
E2F1
E
E2F targets
G2 + M checkpoint
Glycolysis
Autophagy
Mitochondrial biogenesis
Lysosome
RAFi/ERKi over vehicle
RAFi/ERKi over RAFi
RAFi/ERKi over ERKi
Ranking metric E.S.
Genes
Enriched
Depleted
Rank in ordered gene list

Figure S6. The alterations of various signaling pathways upon RAF and ERK inhibition, Related to Figure 3

(A) Kinome-wide chemical proteomics screen by multiplexed kinase inhibitor beads and mass spectrometry (MIB/MS). MIA PaCa-2 cells were treated with the vehicle control DMSO, LY3009120 (RAFi, 0.3 μM), SCH772984 (ERKi, 0.04 μM) alone or in combination for 72 hr. Fold changes of significantly altered kinases (p value ≤ 0.05) are plotted. Each treatment is normalized to its respective vehicle control.

(B) Fold changes (log2) of RNA expression of *ERK* gene targets that drive cancer and *ERK* feedback mechanism regulators. MIA PaCa-2, Pa02C, Pa14C and Pa16C cell lines treated with RAFi + ERKi (0.3 μM and 0.04 μM, respectively) and compared to RAFi (0.3 μM), ERKi (0.04 μM) or the vehicle control (24 hr). Error bars are shown as standard error. p values are from Wald test. Adjusted p values for RAFi + ERKi compared to RAFi = *MYC (*, 0.0161), FOSL1 (**, 0.0018), DUSP4 (****, 1.51E-13), DUSP5 (*, 0.0547), DUSP6 (****, 8.79E-17), DUSP7 (****, 8.90E-07), SPRY2 (***, 0.0002), SPRY4 (****, 5.81E-20), SPRED1 (****, 2.17E-29), SPRED2 (****, 3.05E-06), CCND1 (***, 0.0013), LIF (***, 0.0003).* Adjusted p values for RAFi + ERKi compared to ERKi = *MYC (**, 0.0112), FOSL1 (****, 2.74E-11), DUSP4 (****, 6.39E-19), DUSP5 (**, 0.0019), DUSP6 (****, 3.09E-17), DUSP7 (****, 5.35E-07), SPRY2 (****, 1.80E-12), SPRY4 (****, 1.40E-22), SPRED1 (****, 1.12E-29), SPRED2 (****, 2.67E-07), CCND1 (***, 0.002), LIF (****, 1.96E-05).* Adjusted p values for RAFi + ERKi compared to the vehicle = *MYC (****, 1.13E-05), FOSL1 (****, 5.46E-09), DUSP4 (****, 2.91E-36), DUSP5 (****, 6.44E-19), DUSP6 (****, 1.93E-41), DUSP7 (****, 9.59E-20), SPRY2 (***, 0.0001), SPRY4 (****, 4.05E-52), SPRED1 (****, 2.57E-15), SPRED2 (****, 5.69E-05), CCND1 (**, 0.0039), LIF (****, 6.08E-07).*

(C) Gene set enrichment analysis (GSEA) of hallmark MYC targets. Altered gene expressions are plotted as enrichment scores (E.S.). PDAC cells were treated with LY3009120 (RAFi, 0.3 μM) and SCH772984 (ERKi, 0.04 μM) for 24 hr and normalized to the vehicle control DMSO (left), RAFi (middle), or ERKi (right). Data shown are the average of four PDAC cell lines MIA PaCa-2, Pa02C, Pa14C and Pa16C.

(D) Fold changes (log2) of RNA expression of *IGF1R*, *PTPN11* (SHP2) and *E2F1*. MIA PaCa-2, Pa02C, Pa14C and Pa16C cell lines treated with RAFi + ERKi (0.3 μM and 0.04 μM, respectively) and compared to RAFi (0.3 μM), ERKi (0.04 μM) or the vehicle control (24 hr). Error bars are shown as standard error. p values are from Wald test. Adjusted p values for RAFi + ERKi compared to RAFi = *IGF1R (n.s., 0.9064), PTPN11 (*, 0.0491), E2F1 (***, 0.0005).* Adjusted p values for RAFi + ERKi compared to ERKi = *IGF1R (n.s., 0.8150), PTPN11 (n.s., 0.3391), E2F1 (****, 2.49E-08).* Adjusted *p* values for RAFi + ERKi compared to the vehicle = *IGF1R (n.s., 0.9106), PTPN11 (*, 0.0538), E2F1 (***, 0.0001).* Adjusted p values for RAFi = *IGF1R (n.s., 0.9828), PTPN11 (n.s., 0.2844), E2F1 (n.s.,* 0.0713*).* Adjusted p values for ERKi = *IGF1R (n.s., 0.9916), PTPN11 (n.s., 0.0608), E2F1 (n.s., 0.1325).*

(E) Gene set enrichment analysis (GSEA) of hallmark E2F targets, G2M checkpoint, glycolysis, autophagy, mitochondrial biogenesis, and lysosome. Altered gene expressions are plotted as enrichment scores (E.S.). PDAC cells were treated with LY3009120 (RAFi, 0.3 μM) and SCH772984 (ERKi, 0.04 μM) for 24 hr and normalized to the vehicle control DMSO (left), RAFi (middle), or ERKi (right). Data shown are the average of four PDAC cell lines MIA PaCa-2, Pa02C, Pa14C and Pa16C.

Figure S7

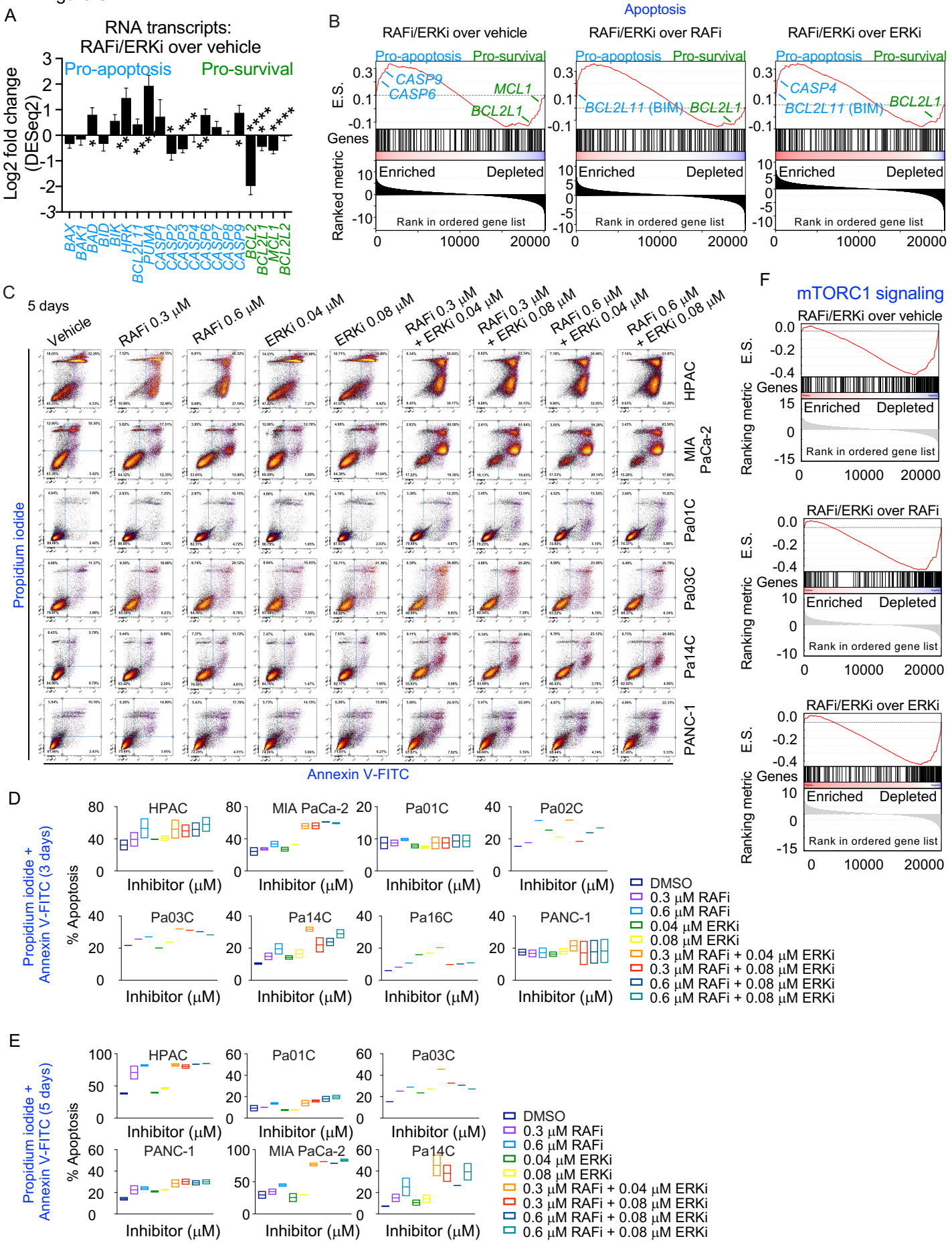
A
RNA transcripts:
RAFi/ERKi over vehicle
Pro-apoptosis
Pro-survival
Log2 fold change (DESeq2)
BAX
BAK1
BAD
BID
BIK
HRK
BCL2L11
PUMA
CASP1
CASP2
CASP3
CASP4
CASP6
CASP7
CASP8
CASP9
BCL2
BCL2L1
MCL1
BCL2L2
B
Apoptosis
RAFi/ERKi over vehicle
RAFi/ERKi over RAFi
RAFi/ERKi over ERKi
Pro-apoptosis
Pro-survival
E.S.
CASP9
CASP6
MCL1
BCL2L1
CASP4
BCL2L11 (BIM)
Genes
Ranked metric
Enriched
Depleted
Rank in ordered gene list
C
5 days
Vehicle
RAFi 0.3 μM
RAFi 0.6 μM
ERKi 0.04 μM
ERKi 0.08 μM
RAFi 0.3 μM + ERKi 0.04 μM
RAFi 0.3 μM + ERKi 0.08 μM
RAFi 0.6 μM + ERKi 0.04 μM
RAFi 0.6 μM + ERKi 0.08 μM
HPAC
MIA PaCa-2
Pa01C
Pa03C
Pa14C
PANC-1
Propidium iodide
Annexin V-FITC
F
mTORC1 signaling
RAFi/ERKi over vehicle
RAFi/ERKi over RAFi
RAFi/ERKi over ERKi
D
Propidium iodide + Annexin V-FITC (3 days)
% Apoptosis
HPAC
MIA PaCa-2
Pa01C
Pa02C
Pa03C
Pa14C
Pa16C
PANC-1
Inhibitor (μM)
DMSO
0.3 μM RAFi
0.6 μM RAFi
0.04 μM ERKi
0.08 μM ERKi
0.3 μM RAFi + 0.04 μM ERKi
0.3 μM RAFi + 0.08 μM ERKi
0.6 μM RAFi + 0.08 μM ERKi
0.6 μM RAFi + 0.08 μM ERKi
E
Propidium iodide + Annexin V-FITC (5 days)
% Apoptosis
HPAC
Pa01C
Pa03C
PANC-1
MIA PaCa-2
Pa14C
Inhibitor (μM)
DMSO
0.3 μM RAFi
0.6 μM RAFi
0.04 μM ERKi
0.08 μM ERKi
0.3 μM RAFi + 0.04 μM ERKi
0.3 μM RAFi + 0.08 μM ERKi
0.6 μM RAFi + 0.08 μM ERKi
0.6 μM RAFi + 0.08 μM ERKi

Figure S7. Concurrent RAF and ERK Inhibition Induces Apoptosis, Related to Figure 4

(A) Fold changes (log2) of RNA expression of pro-apoptosis and pro-survival genes as the averaged values of MIA PaCa-2, Pa02C, Pa14C and Pa16C cell lines treated with RAFi + ERKi (0.3 μM and 0.04 μM, respectively) and compared to the vehicle control DMSO (24 hr). Error bars are shown as standard error. All p values shown are in comparison to the vehicle control. p values are from Wald test. Adjusted p values = *BAX (0.1030), BAK1 (0.6372), BAD (*, 0.0195), BID (0.4139), BIK (0.0835), HRK (**, 0.0016), BCL2L11 (0.1258), PUMA/BBC3 (****, 5.78E-05), CASP1 (0.4693), CASP2 (*, 0.0170), CASP3 (***, 0.0008), CASP4 (0.9750), CASP6 (**, 0.0050), CASP7 (0.2896), CASP8 (0.8912), CASP9 (*, 0.0158), BCL2 (****, 2.83E-07), BCL2L1 (*, 0.0171), MCL1 (****, 1.13E-05), BCL2L2 (0.8906).*

(B) GSEA of hallmark apoptosis signaling. Altered gene expressions are plotted as E.S. (enrichment score) PDAC cells were treated with LY3009120 (RAFi, 0.3 μM) and SCH772984 (ERKi, 0.04 μM) for 24 hr and normalized to the vehicle control (left), RAFi (middle), or ERKi (right). Data are the average of four PDAC cell lines MIA PaCa-2, Pa02C, Pa14C and Pa16C.

(C) Related to Figure 4D. Representative images of percent apoptosis induced by the vehicle control, LY3009120 (RAFi, 0.3 or 0.6 μM), SCH772984 (ERKi, 0.04 or 0.08 μM) or the combinations (120 hr). FACS analysis of Annexin-V/propidium iodide labeled cells was used to measure apoptosis.

(D) Quantitation of percent apoptosis of the indicated *KRAS* mutant PDAC cells treated with the vehicle control, LY3009120 (RAFi, 0.3 or 0.6 μM), SCH772984 (ERKi, 0.04 or 0.08 μM) or the combinations for 72 hr or

(E) 120 hr. FACS analysis of Annexin V-FITC/propidium iodide-labeled cells was used to measure apoptosis.

(F) Gene set enrichment analysis (GSEA) of hallmark mTORC1 signaling. Altered gene expressions are plotted as enrichment scores (E.S.). PDAC cells were treated with LY3009120 (RAFi, 0.3 μM) and SCH772984 (ERKi, 0.04 μM) for 24 hr and normalized to the vehicle control DMSO (upper graph), RAFi (middle graph), or ERKi (lower graph). Data are the average of four PDAC cell lines MIA PaCa-2, Pa02C, Pa14C and Pa16C.

Figure S8

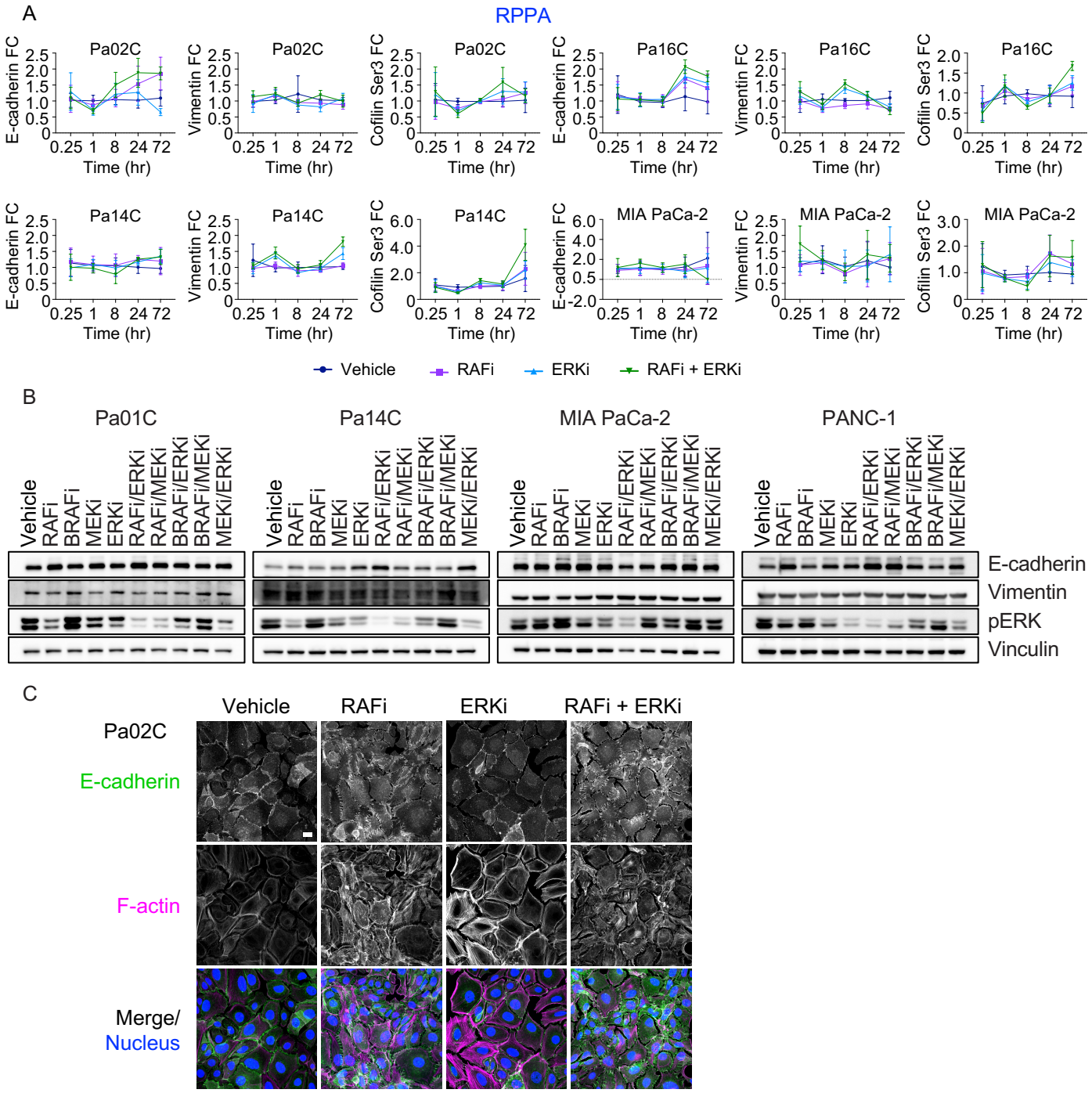

Figure S8. Combined RAF and ERK Inhibition Causes Mesenchymal to Epithelial Transition, Related to Figure 5

(A) RPPA. PDAC cells were treated with the vehicle control, LY3009120 (RAFi, 0.3 μM), SCH772984 (ERKi, 0.04 μM) or the combination for 15 min, 1, 8, 24 and 72 hr. The alterations of protein expression of E-cadherin (epithelial marker), vimentin (mesenchymal marker) and phosphorylated cofilin (actin polymerization) were plotted over time. Data are the mean average of five independent experiments. Error bars are shown as ±S.E.M. FC: fold change.

(B) Pa01C, Pa14C, MIA PaCa-2 and PANC-1 cells were treated with LY3009120 (RAFi, 0.3 μM), vemurafenib (BRAFi, 1 μM), trametinib (MEKi, 0.5 nM) or SCH772984 (ERKi, 0.04 μM) alone or in combination as indicated (120 hr). Cell lysates were immunoblotted to determine the levels of E-cadherin, vimentin, phosphorylated ERK and vinculin.

(C) Related to Figure 5C. Representative images (the same representative image shown in Figure 5C as E-cadherin and nucleus merge) of Pa02C cells treated with vehicle control, LY3009120 (RAFi, 0.3 μM), SCH772984 (ERKi, 0.04 μM) or the combination to visualize F-actin (shown alone or merged with E-cadherin and DAPI) expression and distribution (72 hr). Scale bar, 20 μm.

Figure S9

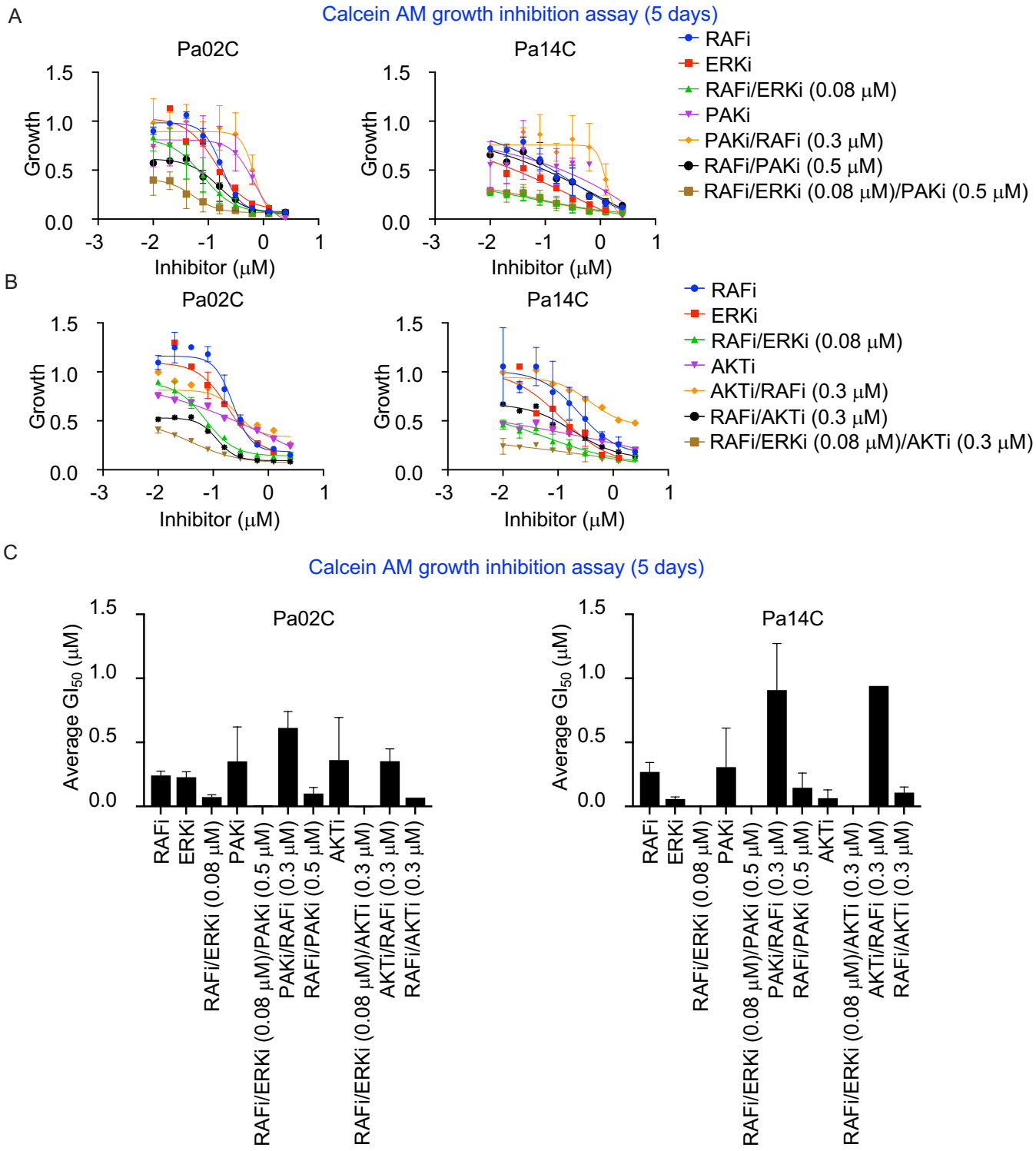

Figure S9. Concurrent PAK or AKT Inhibition Enhances RAFi/ERKi Activity, Related to Figure 6

(A) Pa02C and Pa14C cell lines were treated with LY3009120 (RAFi, 0.01-2.5 μM), SCH772984 (ERKi, 0.08-1.25 μM), FRAX597 (PAKi, 0.01-2.5 μM) alone or the combination with a constant concentration of a second or third inhibitor as indicated. Proliferation was measured after 120 hr by Calcein AM cell viability assay. Data are the mean average of two independent experiments. Error bars are shown as ±S.E.M.

(B) Pa02C and Pa14C cell lines were treated with LY3009120 (RAFi, 0.01-2.5 μM), SCH772984 (ERKi, 0.08-1.25 μM), MK2206 (AKTi, 0.01-2.5 μM) alone or the combination with a constant concentration of a second or third inhibitor as indicated. Proliferation was measured after 120 hr by Calcein AM cell viability assay. Data are the mean average of two independent experiments. Error bars are shown as ±S.E.M.

(C) Average GI_{50} values for the data described in Figures S9A and S9B.

Figure S10

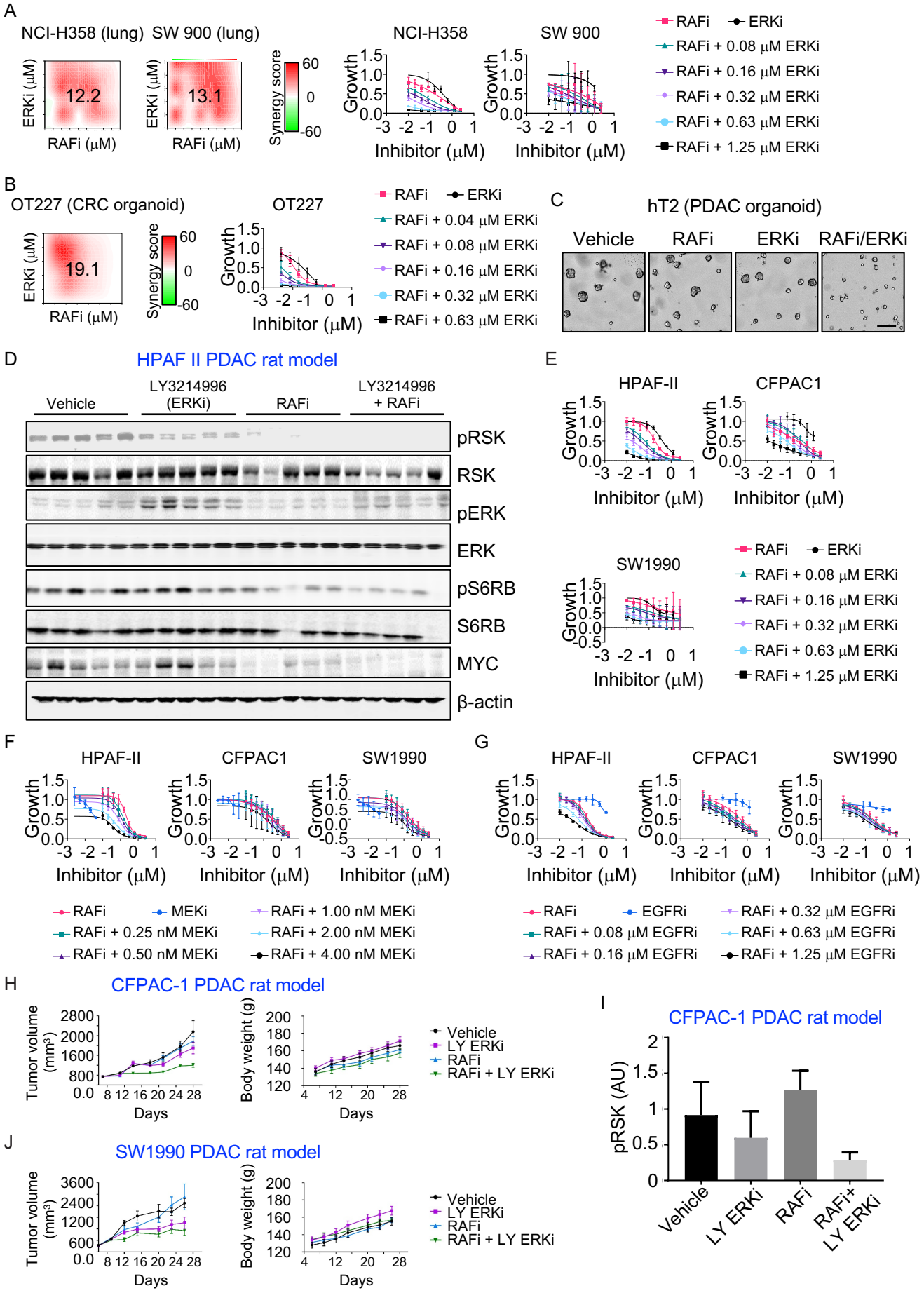
A
NCI-H358 (lung)
SW 900 (lung)
ERKi (μM)
RAFi (μM)
12.2
13.1
Synergy score
60
0
-60
NCI-H358
SW 900
Growth
Inhibitor (μM)
RAFi
ERKi
RAFi + 0.08 μM ERKi
RAFi + 0.16 μM ERKi
RAFi + 0.32 μM ERKi
RAFi + 0.63 μM ERKi
RAFi + 1.25 μM ERKi
B
OT227 (CRC organoid)
19.1
OT227
RAFi + 0.04 μM ERKi
C
hT2 (PDAC organoid)
Vehicle
RAFi
ERKi
RAFi/ERKi
D
HPAF II PDAC rat model
Vehicle
LY3214996 (ERKi)
RAFi
LY3214996 + RAFi
pRSK
RSK
pERK
ERK
pS6RB
S6RB
MYC
β-actin
E
HPAF-II
CFPAC1
SW1990
F
MEKi
RAFi + 1.00 nM MEKi
RAFi + 0.25 nM MEKi
RAFi + 2.00 nM MEKi
RAFi + 0.50 nM MEKi
RAFi + 4.00 nM MEKi
G
EGFRi
RAFi + 0.32 μM EGFRi
RAFi + 0.08 μM EGFRi
RAFi + 0.63 μM EGFRi
RAFi + 0.16 μM EGFRi
RAFi + 1.25 μM EGFRi
H
CFPAC-1 PDAC rat model
Tumor volume (mm³)
Body weight (g)
Days
Vehicle
LY ERKi
RAFi
RAFi + LY ERKi
I
CFPAC-1 PDAC rat model
pRSK (AU)
Vehicle
LY ERKi
RAFi
RAFi+ LY ERKi
J
SW1990 PDAC rat model

Figure S10. Concurrent RAF and ERK Inhibition Synergistically Impairs KRAS mutant cancer growth *in vitro* and *in vivo*, Related to Figure 7

(A) *KRAS* mutant lung cancer cell lines NCI-H358 and SW 900 were treated with the vehicle control (lower left of each graph), LY3009120 (RAFi, 0.01-2.5 μM) and SCH772984 (ERKi, 0.08-1.25 μM) alone or in combination for 120 hr. Proliferation was measured by Calcein AM cell viability assay. Representative bliss synergy score heatmaps for three independent experiments is shown (left). Red (synergy), green (antagonism), white (no effect). Averaged synergy scores presented on the synergy heatmaps. Averaged dose response curves of three independent experiments are shown (right). Error bars are ±S.E.M.

(B) *KRAS* mutant CRC organoids OT227 were treated with the vehicle control (lower left of each graph), LY3009120 (RAFi, 0.01-2.5 μM) and SCH772984 (ERKi, 0.04-0.63 μM) alone or in combination for 5 days. Proliferation was measured by CellTiter-Glo 3D cell viability assay. Dose response curves and bliss synergy scores were calculated and represented as in Figure S10A.

(C) Representative images of PDAC organoid hT2 treated with the vehicle control DMSO, LY3009120 (RAFi, 0.16 μM), SCH772984 (ERKi, 0.04 μM) alone or in combination. Scale bar = 200 μm.

(D) Tumors from HPAF-II PDAC rat (n = 5 for each condition) were harvested at day 21. Tumor lysates were immunoblotted to determine levels of phosphorylated and total RSK, phosphorylated and total ERK, phosphorylated ribosomal protein S6 (S240/244), total ribosomal protein S6, MYC and B-actin (control for total protein).

(E) The indicated PDAC cell lines were treated with LY3009120 (RAFi, 0.01-2.5 μM) and SCH772984 (ERKi, 0.08-1.25 μM) alone or in combination for 120 hr. Proliferation was measured by Calcein AM cell viability assay. Averaged dose response curves of three independent experiments are shown. Error bars are ±S.E.M.

(F) The indicated PDAC cell lines were treated with LY3009120 (RAFi, 0.01-2.5 μM) and trametinib (MEKi, 0.25 - 4.00 μM) alone or in combination for 120 hr. Proliferation was measured by Calcein AM cell viability assay. Averaged dose response curves of three independent experiments are shown. Error bars are ±S.E.M.

(G) The indicated PDAC cell lines were treated with LY3009120 (RAFi, 0.01-2.5 μM) and erlotinib (EGFRi, 0.08-1.25 μM) alone or in combination for 120 hr. Proliferation was measured by Calcein AM cell viability assay. Averaged dose response curves of three independent experiments are shown. Error bars are ±S.E.M.

(H) Relative tumor volume of the NIH nude rats with implanted CFPAC-1 cells were treated with LY3009120 (RAFi, 20 mpk, BID) alone or in combination with ERK1/2 inhibitor LY3214996 (LY ERKi, 10 mpk, QD) for 36 days (left). Body weight changes are shown (right).

(I) Quantitation of the western blot analysis to determine the levels of phosphorylated RSK of tumor lysates (n = 5 animals per group).

(J) Relative tumor volume of the NIH nude rats with implanted SW 1990 cells were treated with LY3009120 (RAFi, 20 mpk, BID) alone or in combination with ERK1/2 inhibitor LY3214996 (LY ERKi, 10 mpk, QD) for 36 days (left). Body weight changes are shown (right).