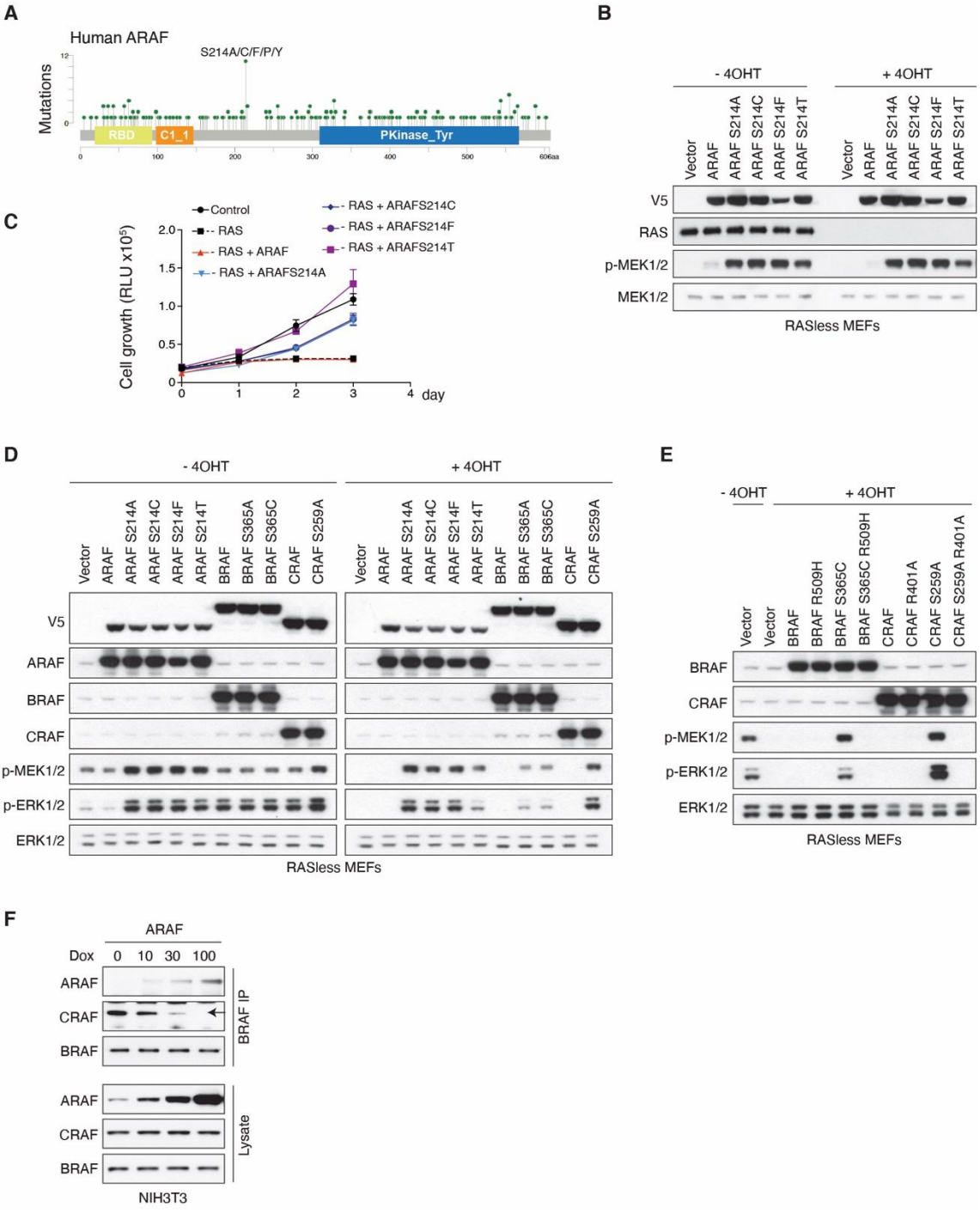
A
Human ARAF
S214A/C/F/P/Y
Mutations
RBD
C1_1
PKinase_Tyr
606aa
B
- 4OHT
+ 4OHT
Vector
ARAF
ARAF S214A
ARAF S214C
ARAF S214F
ARAF S214T
V5
RAS
p-MEK1/2
MEK1/2
RASless MEFs
C
Control
RAS
RAS + ARAF
RAS + ARAFS214A
RAS + ARAFS214C
RAS + ARAFS214F
RAS + ARAFS214T
Cell growth (RLU x10^5)
day
D
BRAF
BRAF S365A
BRAF S365C
CRAF
CRAF S259A
ARAF
BRAF
CRAF
p-MEK1/2
p-ERK1/2
ERK1/2
RASless MEFs
E
BRAF R509H
BRAF S365C R509H
CRAF R401A
CRAF S259A R401A
RASless MEFs
F
ARAF
Dox
0
10
30
100
BRAF IP
Lysate
NIH3T3

Figure S1. ARAF S214 mutants signal as RAS-independent dimers but do not result in feedback inhibition of cellular RAS-GTP, Related to Figure 1.

(**A**) Schematic representation of ARAF mutations identified in human cancers from cBioportal.

(**B**) K-Ras^{lox} MEF cells stably expressing doxycycline-inducible WT ARAF or the indicated mutants were grown in medium without or with 1 μM 4-OHT for a week to generate isogenic cells expressing or lacking K-RAS Cells. Cells were then treated with 100 ng/ml doxycycline for 48 hours. ARAF proteins were immunoprecipitated with anti-V5 agarose. The *in vitro* kinase assay was performed with kinase-dead MEK1 (K97R).

(**C**) K-Ras^{lox} MEF cells stably expressing doxycycline-inducible ARAF variants were grown in medium without or with 1 μM 4-OHT for a week. Cells were treated with 100 ng/ml doxycycline for 24 hours and then plated in 96 well plates. Cell growth rates were measured. Bars, mean±SD triplicate of experiments.

(**D** and **E**) K-Ras^{lox} MEF cells expressing or lacking K-RAS were transfected with indicated plasmids and harvested 24 hours after transfection. ERK pathway activity was examined by western blot.

(**F**) NIH3T3 cells expressing doxycycline-inducible ARAF were treated with doxycycline for 48 hours. Cells were collected and endogenous BRAF was immunoprecipitated.

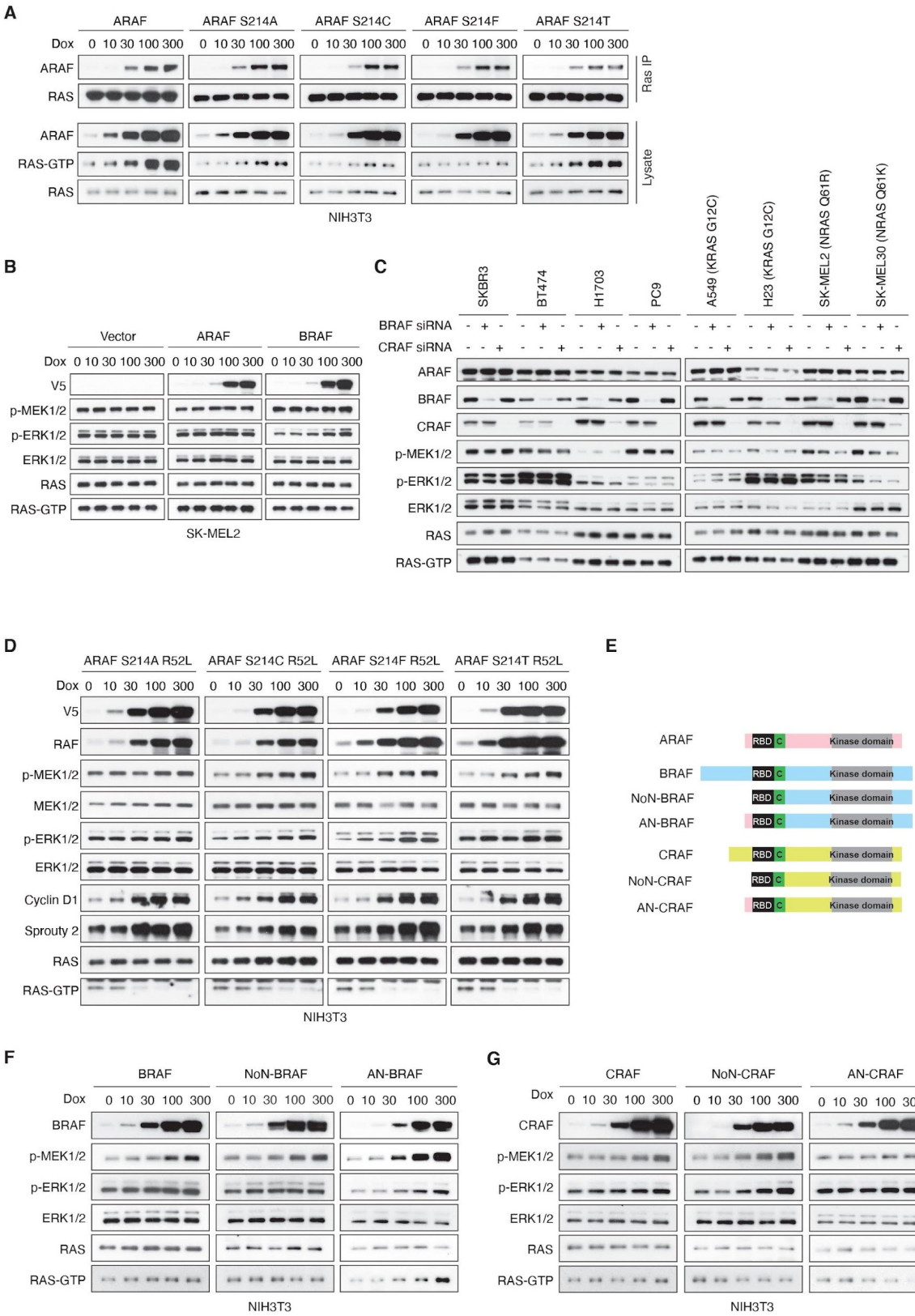
A
ARAF
ARAF S214A
ARAF S214C
ARAF S214F
ARAF S214T
Dox 0 10 30 100 300
ARAF
RAS
Ras IP
ARAF
RAS-GTP
RAS
Lysate
NIH3T3
B
Vector
ARAF
BRAF
Dox 0 10 30 100 300
V5
p-MEK1/2
p-ERK1/2
ERK1/2
RAS
RAS-GTP
SK-MEL2
C
SKBR3
BT474
H1703
PC9
A549 (KRAS G12C)
H23 (KRAS G12C)
SK-MEL2 (NRAS Q61R)
SK-MEL30 (NRAS Q61K)
BRAF siRNA
CRAF siRNA
ARAF
BRAF
CRAF
p-MEK1/2
p-ERK1/2
ERK1/2
RAS
RAS-GTP
D
ARAF S214A R52L
ARAF S214C R52L
ARAF S214F R52L
ARAF S214T R52L
Dox 0 10 30 100 300
V5
RAF
p-MEK1/2
MEK1/2
p-ERK1/2
ERK1/2
Cyclin D1
Sprouty 2
RAS
RAS-GTP
NIH3T3
E
ARAF
BRAF
NoN-BRAF
AN-BRAF
CRAF
NoN-CRAF
AN-CRAF
RBD
C
Kinase domain
F
BRAF
NoN-BRAF
AN-BRAF
Dox 0 10 30 100 300
BRAF
p-MEK1/2
p-ERK1/2
ERK1/2
RAS
RAS-GTP
NIH3T3
G
CRAF
NoN-CRAF
AN-CRAF
Dox 0 10 30 100 300
CRAF
p-MEK1/2
p-ERK1/2
ERK1/2
RAS
RAS-GTP
NIH3T3

Figure S2. ARAF increases GTP-bound WT RAS in a kinase-independent manner, Related to Figure 2.

(**A**) NIH3T3 cells expressing doxycycline-inducible WT or mutant ARAF were treated with different doses of doxycycline for 48 hours. Cell lysates were then subjected to endogenous RAS immunoprecipitation and RAS-GTP assay.

(**B**) SK-MEL-2 cells expressing empty vector, doxycycline-inducible ARAF or BRAF were treated with doxycycline for 48 hours. ERK pathway activity and RAS-GTP levels were examined by western blot.

(**C**) SKBR3, BT474, H1703, PC9, A549, H23, SK-MEL-2 and SK-MEL30 cells were transfected with scramble, BRAF or CRAF siRNA. Cells were collected 48 hours after transfection.

(**D**) NIH3T3 cells expressing doxycycline-inducible ARAF mutants were treated with doxycycline for 48 hours.

(**E**) Schematic representation of functional domains of RAF proteins and engineered proteins with different N-terminus: NoN-BRAF, 154 amino acids of BRAF N-terminus is deleted; AN-BRAF, 154 amino acids of BRAF N-terminus is replaced by 18 amino acids from ARAF N-terminus; NoN-CRAF, 55 amino acids of CRAF N-terminus is deleted; AN-CRAF, 55 amino acids of CRAF N-terminus is replaced by 18 amino acids from ARAF N-terminus.

(**F** and **G**) NIH3T3 cells expressing doxycycline-inducible WT or engineered BRAF or CRAF were treated with doxycycline for 48 hours.

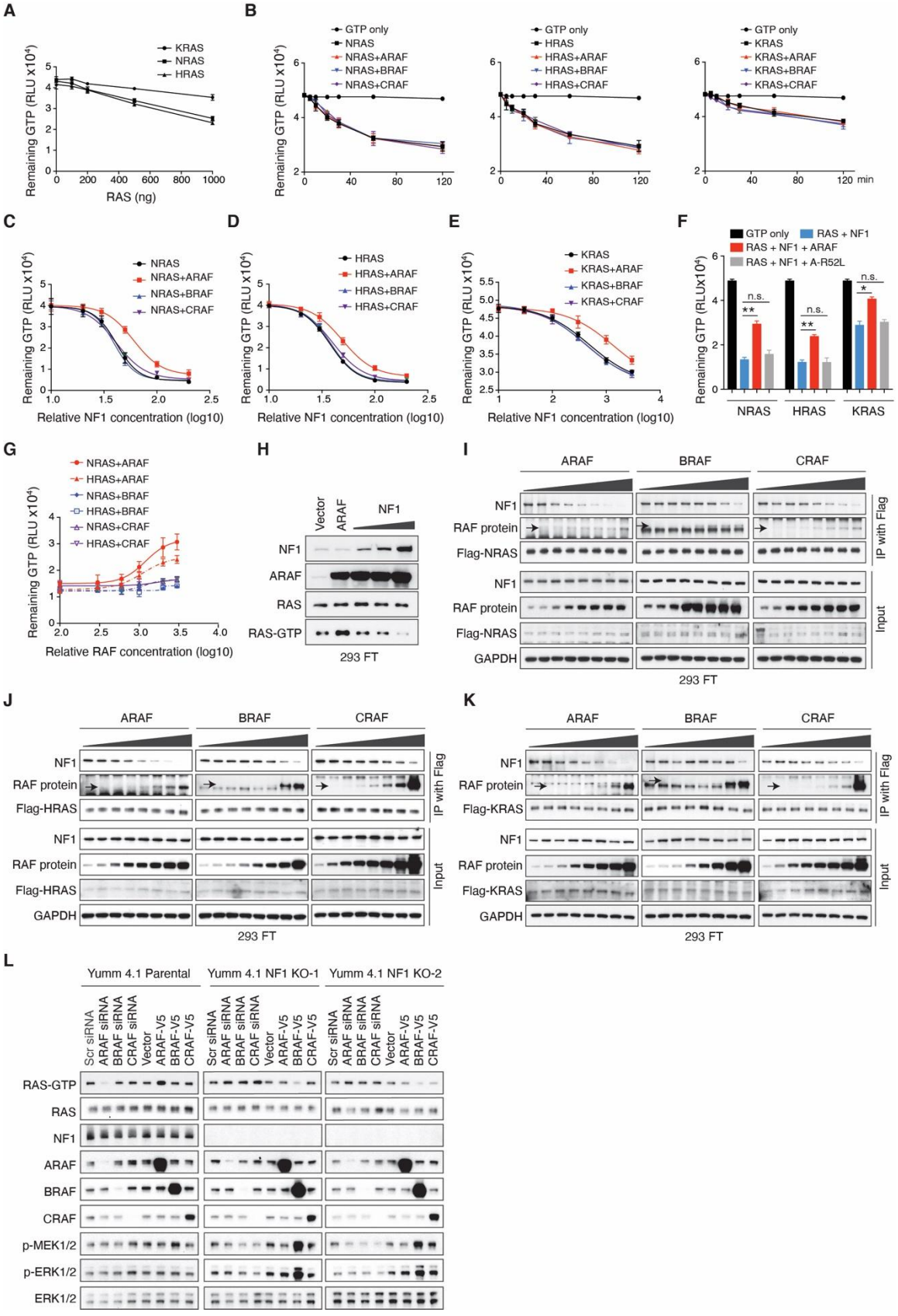
A
KRAS
NRAS
HRAS
Remaining GTP (RLU x10⁴)
RAS (ng)
B
GTP only
NRAS
NRAS+ARAF
NRAS+BRAF
NRAS+CRAF
HRAS
HRAS+ARAF
HRAS+BRAF
HRAS+CRAF
KRAS
KRAS+ARAF
KRAS+BRAF
KRAS+CRAF
min
C
D
E
Relative NF1 concentration (log10)
F
GTP only
RAS + NF1
RAS + NF1 + ARAF
RAS + NF1 + A-R52L
Remaining GTP (RLUx10⁴)
n.s.
NRAS
HRAS
KRAS
G
Relative RAF concentration (log10)
H
Vector
ARAF
NF1
RAS
RAS-GTP
293 FT
I
ARAF
BRAF
CRAF
RAF protein
Flag-NRAS
GAPDH
IP with Flag
Input
J
Flag-HRAS
K
Flag-KRAS
L
Yumm 4.1 Parental
Yumm 4.1 NF1 KO-1
Yumm 4.1 NF1 KO-2
Scr siRNA
ARAF siRNA
BRAF siRNA
CRAF siRNA
Vector
ARAF-V5
BRAF-V5
CRAF-V5
p-MEK1/2
p-ERK1/2
ERK1/2

Figure S3. ARAF increases RAS-GTP by competing with RASGAP NF1 for binding to RAS, Related to Figure 3.

(**A**) GTP hydrolysis reactions were assembled with purified N-, H-, or K-RAS. Reactions were incubated for 1 hour and luminescence was recorded. Bars, mean±SD biological triplicate of experiments.

(**B**) GTP hydrolysis reactions were assembled with 0.5 μg (1 μM) purified RAS and 2 μg A-, B- or C-RAF proteins (~1 μM). Bars, mean±SD biological triplicate of experiments.

(**C**-**E**) GTP hydrolysis reactions were assembled with 0.5 μg (1 μM) purified N- (C), H- (D), or K-RAS (E) and different amounts of NF1-333 proteins in the present of 2 μg A-, B- or C-RAF proteins (~1 μM). Reactions were incubated for 30 minutes at room temperature. Bars, mean±SD biological triplicate of experiments.

(**F**) GTP hydrolysis reactions were assembled with 0.5 μg (1 μM) RAS, NF1-333 (50 ng/ 50 nM for N- or H-RAS, 500 ng/500 nM for KRAS) and 3 μg (1.8 μM) WT ARAF or ARAF R52L proteins. Bars, mean±SD biological triplicate of experiments. ** $p<0.01$, * $p<0.05$, n.s. not significant, two-tailed Student's t test

(**G**) GTP hydrolysis reactions were assembled with 0.5 μg (1 μM) purified N- or H-RAS and 50 ng (50 nM) NF1-333 with different amounts of A-, B- or C-RAF proteins (from 60 nM to 1.8 μM). Reactions were incubated for 30 minutes at room temperature. Bars, mean±SD biological triplicate of experiments.

(**H**) 293FT cells transfected with WT ARAF and different amounts of NF1 constructs were collected 24 hours after transfection. Lysates were subjected to RAS-GTP assay.

(**I**-**K**) 293FT cells transfected with FLAG tagged N- (I), H- (J) or K-RAS (K), NF1 and different amounts of RAF constructs were collected 24 hours after transfection. NF1 was co-immunoprecipitated with RAS.

(**L**) Parental or NF1 knockout Yumm 4.1 cells were transfected with the indicated constructs or siRNAs. Cells were collected 48 hours after transfection.

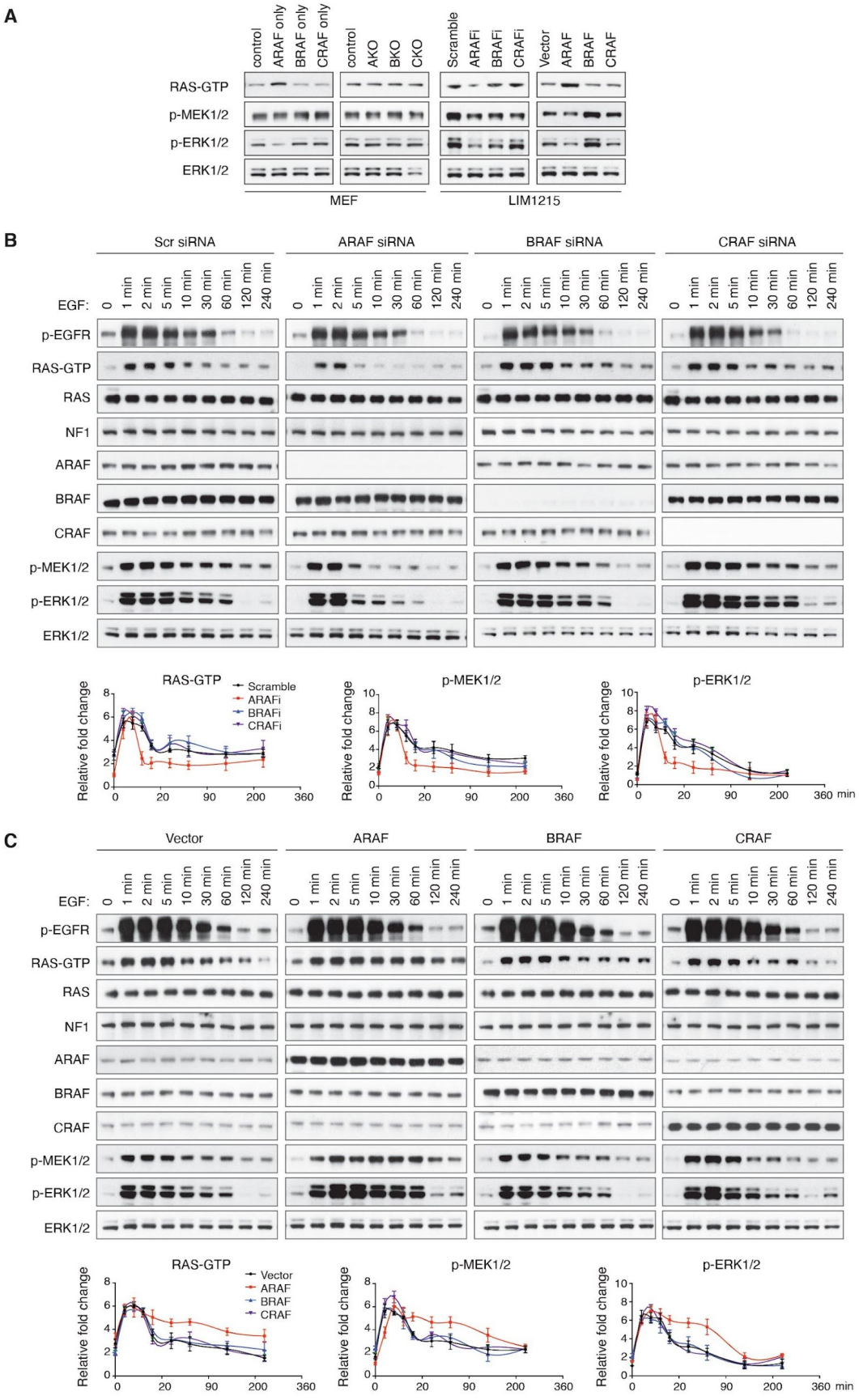
A
control
ARAF only
BRAF only
CRAF only
control
AKO
BKO
CKO
Scramble
ARAFi
BRAFi
CRAFi
Vector
ARAF
BRAF
CRAF
RAS-GTP
p-MEK1/2
p-ERK1/2
ERK1/2
MEF
LIM1215
B
Scr siRNA
ARAF siRNA
BRAF siRNA
CRAF siRNA
EGF:
0
1 min
2 min
5 min
10 min
30 min
60 min
120 min
240 min
p-EGFR
RAS-GTP
RAS
NF1
ARAF
BRAF
CRAF
p-MEK1/2
p-ERK1/2
ERK1/2
RAS-GTP
Scramble
ARAFi
BRAFi
CRAFi
p-MEK1/2
p-ERK1/2
Relative fold change
0
2
4
6
8
10
0
20
90
200
360
360 min
C
Vector
ARAF
BRAF
CRAF
EGF:
0
1 min
2 min
5 min
10 min
30 min
60 min
120 min
240 min
p-EGFR
RAS-GTP
RAS
NF1
ARAF
BRAF
CRAF
p-MEK1/2
p-ERK1/2
ERK1/2
RAS-GTP
Vector
ARAF
BRAF
CRAF
p-MEK1/2
p-ERK1/2
Relative fold change
0
2
4
6
8
10
0
20
90
200
360
360 min

Figure S4. The level of ARAF expression is a determinant of the duration of RAS activation in cells, Related to Figure 4.

(**A**) Signaling activity of MEF cells or LIM1215 cells transfected with indicated siRNAs or constructs were shown.

(**B** and **C**) LIM1215 cells transfected with indicated siRNAs (B) or constructs (C) were treated with 100 ng/ml recombinant EGF for the indicated time points. Signaling activity and RAS-GTP levels were examined by western blot. Lower panel, RAS-GTP, p-MEK and p-ERK levels were quantified and normalized to basal levels. Bars, mean±SD biological triplicate of experiments.

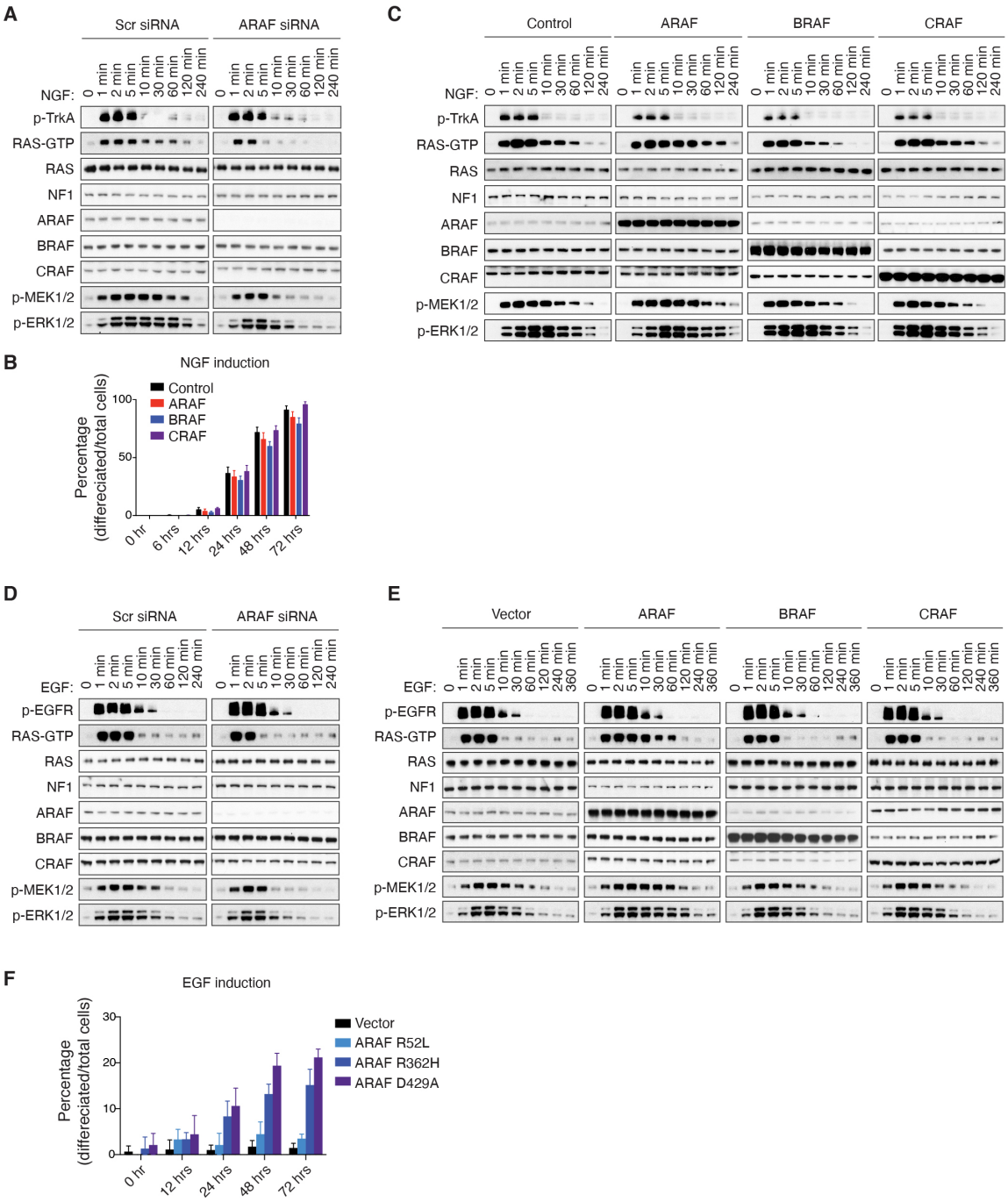
A
Scr siRNA
ARAF siRNA
NGF:
0 1 min 2 min 5 min 10 min 30 min 60 min 120 min 240 min
p-TrkA
RAS-GTP
RAS
NF1
ARAF
BRAF
CRAF
p-MEK1/2
p-ERK1/2
B
NGF induction
Percentage (differeciated/total cells)
Control
ARAF
BRAF
CRAF
0 hr
6 hrs
12 hrs
24 hrs
48 hrs
72 hrs
C
Control
ARAF
BRAF
CRAF
D
EGF:
p-EGFR
E
Vector
F
EGF induction
Vector
ARAF R52L
ARAF R362H
ARAF D429A

Figure S5. Expression of ARAF influences the biological consequences of ligand activated RAS signaling, Related to Figure 5.

(**A**) PC12 cells were transfected with indicated siRNAs for 48 hours and then treated with 50 ng/ml recombinant NGF for the indicated time points. ERK pathway activity and RAS-GTP levels were examined by western blot.

(**B** and **C**) PC12 cells expressing doxycycline-inducible A-, B- or C-RAF were treated with 100 ng/ml doxycycline for 24 hours. Cells were then treated with 50 ng/ml recombinant NGF. Percentage of differentiated cells was quantified at the indicated time points. Error bars, mean±SD biological triplicate of experiments (B). ERK pathway activity and RAS-GTP levels were examined by western blot (C).

(**D**) PC12 cells were transfected with indicated siRNAs for 48 hours and then treated with 100 ng/ml recombinant EGF for the indicated time points.

(**E**) PC12 cells expressing doxycycline-inducible A-, B- or C-RAF were treated with 100 ng/ml doxycycline for 24 hours. Cells were then treated with 100 ng/ml recombinant EGF for the indicated times.

(**F**) PC12 cells expressing doxycycline-inducible ARAF mutants were treated with 100 ng/ml doxycycline for 24 hours. Cells were then treated with 100 ng/ml recombinant EGF. Percentage of differentiated cells was quantified at the indicated time points. Error bars, mean±SD biological triplicate of experiments.

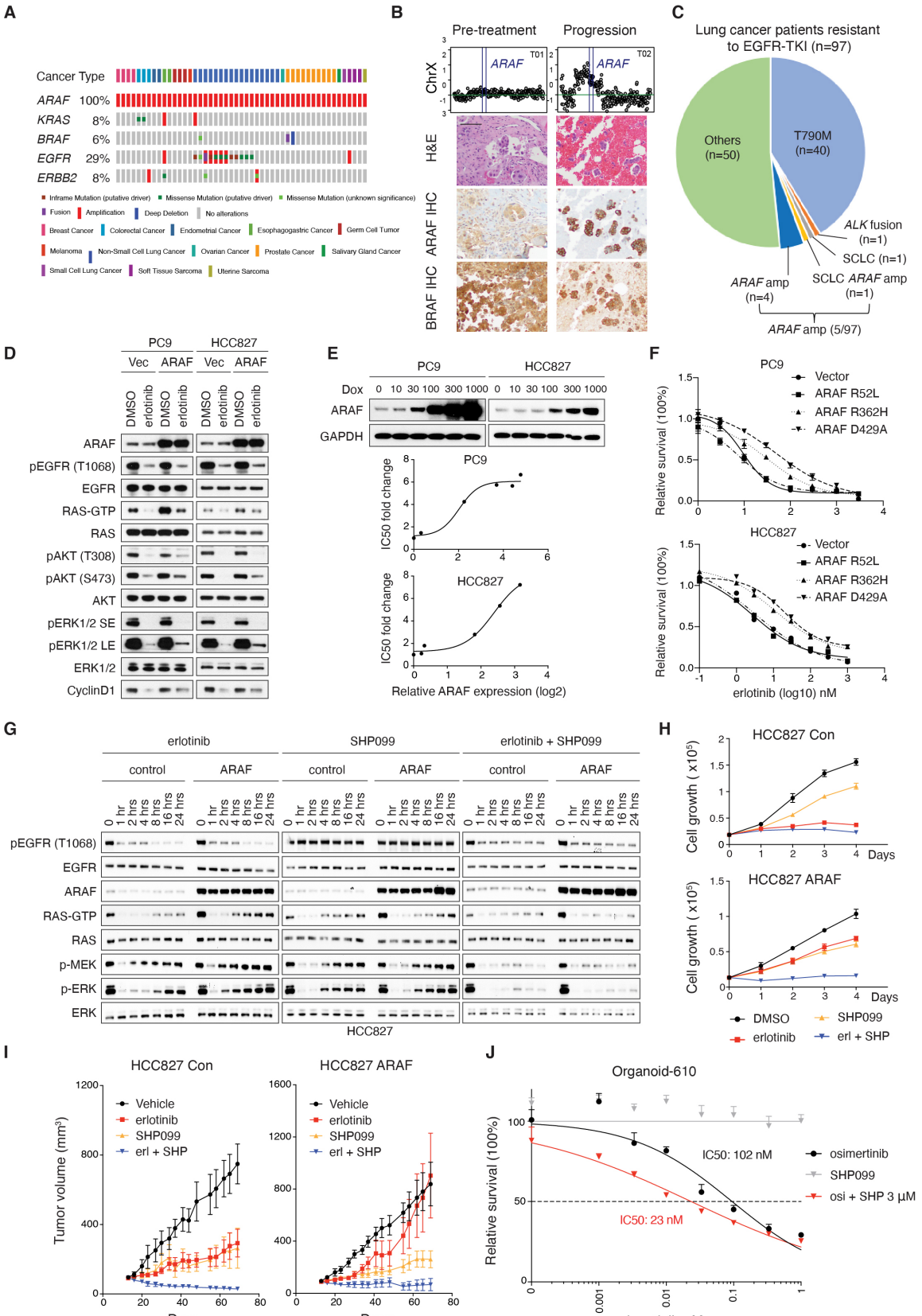
A
Cancer Type
ARAF 100%
KRAS 8%
BRAF 6%
EGFR 29%
ERBB2 8%
Inframe Mutation (putative driver)
Missense Mutation (putative driver)
Missense Mutation (unknown significance)
Fusion
Amplification
Deep Deletion
No alterations
Breast Cancer
Colorectal Cancer
Endometrial Cancer
Esophagogastric Cancer
Germ Cell Tumor
Melanoma
Non-Small Cell Lung Cancer
Ovarian Cancer
Prostate Cancer
Salivary Gland Cancer
Small Cell Lung Cancer
Soft Tissue Sarcoma
Uterine Sarcoma
B
Pre-treatment
Progression
ChrX
ARAF
T01
T02
H&E
ARAF IHC
BRAF IHC
C
Lung cancer patients resistant to EGFR-TKI (n=97)
Others (n=50)
T790M (n=40)
ALK fusion (n=1)
SCLC (n=1)
SCLC ARAF amp (n=1)
ARAF amp (n=4)
ARAF amp (5/97)
D
PC9
HCC827
Vec
ARAF
DMSO
erlotinib
ARAF
pEGFR (T1068)
EGFR
RAS-GTP
RAS
pAKT (T308)
pAKT (S473)
AKT
pERK1/2 SE
pERK1/2 LE
ERK1/2
CyclinD1
E
PC9
HCC827
Dox
0 10 30 100 300 1000
ARAF
GAPDH
IC50 fold change
Relative ARAF expression (log2)
F
PC9
HCC827
Vector
ARAF R52L
ARAF R362H
ARAF D429A
Relative survival (100%)
erlotinib (log10) nM
G
erlotinib
SHP099
erlotinib + SHP099
control
ARAF
0 1 hr 2 hrs 4 hrs 8 hrs 16 hrs 24 hrs
pEGFR (T1068)
EGFR
ARAF
RAS-GTP
RAS
p-MEK
p-ERK
ERK
HCC827
H
HCC827 Con
HCC827 ARAF
Cell growth (x10^5)
Days
DMSO
erlotinib
SHP099
erl + SHP
I
HCC827 Con
HCC827 ARAF
Vehicle
erlotinib
SHP099
erl + SHP
Tumor volume (mm^3)
Days
J
Organoid-610
IC50: 102 nM
IC50: 23 nM
osimertinib
SHP099
osi + SHP 3 μM
Relative survival (100%)
osimertinib μM

Figure S6. ARAF overexpression causes acquired resistance of EGFR mutant lung cancers to EGFR inhibitors, Related to Figure 6.

(**A**) Oncoprint showing *ARAF* amplification with concurrent genetic alterations of *KRAS*, *BRAF*, *EGFR* and *ERRB2* in patient tumors (http://cbioportal.org).

(**B**) Copy number plots for pre-treatment and progression specimen from patient 1 with probes corresponding to *ARAF*. The Y-axis indicates log2 (tumor/normal) copy number (upper panel). Immunohistochemical staining of ARAF and BRAF in pre- and progression specimens (lower panel). Scale bar, 100 μm.

(**C**) EGFR-TKI resistant lung cancer patients with acquired *ARAF* amplification from a Japanese cohort.

(**D**) PC9 and HCC827 cells expressing vector or doxycycline-inducible ARAF were treated with 100ng/ml doxycycline for 24 hours. Cells were then treated with 100 nM erlotinib for 24 hours. Signaling activity and RAS-GTP levels were examined by western blot.

(**E**) PC9 and HCC827 cells expressing doxycycline-inducible ARAF were treated with different doses of doxycycline for 24 hours. Cells were then seeded in 96 well plates at a density of 2,000 cells/well and treated with erlotinib for 72 hours. Fold changes of erlotinib IC_{50} were indicated upon increasing expression of ARAF.

(**F**) PC9 or HCC827 cells expressing vector or doxycycline-inducible ARAF mutants were seeded in 96 well plates at a density of 2,000 cells/well and supplemented with 100 ng/ml doxycycline. After 24 hours induction, cells were treated with erlotinib for 72 hours. Cell survival was normalized to untreated controls. Drug concentrations inducing 50% inhibition in survival (IC_{50} nmol/L) are indicated. Bars, mean±SD biological triplicate of experiments.

(**G**) HCC827 cells expressing vector or doxycycline-inducible ARAF were treated with 100 ng/ml doxycycline for 24 hours. Cells were then treated with 100 nM erlotinib, 3 μM SHP099 or in combination.

(**H**) HCC827 cells expressing vector or doxycycline-inducible ARAF were seeded in 96 well plates at a density of 2,000 cells/well and supplemented with 100 ng/ml doxycycline. Cells were then treated with 100 nM erlotinib, 3 μM SHP099, or in combination. Bars, mean±SD triplicate of experiments.

(**I**) HCC827 cells expressing doxycycline-inducible ARAF were subcutaneously injected into nude mice. Mice were fed either normal or doxycycline containing food. Erlotinib was given at 12.5 mg/kg once a day, SHP099 at 75 mg/kg once a day. Bars, mean±SEM, n=5.

(**J**) Organoid-610 cells were grown in 3D culture system. Cells were treated with osimertinib, SHP099, or in combination for 72 hours. Cell survival was normalized to untreated controls. Drug concentrations inducing 50% inhibition in survival (IC_{50} nmol/L) are indicated. Bars, mean±SD triplicate of experiments.

Table S1. Genetic alterations of NSCLC patient, Related to Figure 6.

1 Metastasis (lung); 2 Metastasis (bone)

Tumors	Mutations	Tumors	Gene
1 2	EGFR *L858R*	1 2	EGFR AMP
1 2	YAP1 *F385L*	1 2	FGFR4 AMP
1	MAPK3 *R96H*	2	ETV1 AMP
1 2	DOT1L *I232N*	2	PIK3R1 DeepDel
		2	RAC1 AMP
		2	CARD11 AMP
		2	PDGFRB AMP
		1 2	NPM1 AMP
		1 2	NSD1 AMP
		2	CSF1R AMP
		2	PMS2 AMP
		2	KDM6A AMP
		2	RBM10 AMP
		2	ARAF AMP

Table S2. Genetic alterations of PDX-138, Related to Figure 6.

Mutations	Copy number alterations
EGFR *E746_A750del*	BRCA2 DeepDel
TP53 *R249T*	CDKN2A DeepDel
ASXL2 *X620_splice*	FOXA1 AMP
AXL *N699S*	NKX2-1 AMP
GRIN2A *D667G*	LATS2 DeepDel
MAP3K13 *M233V*	MCL1 AMP
PJA2 *PJA2-BRAF fusion*	MYC AMP
	RB1 DeepDel
	CCNE1 AMP
	CDKN2B DeepDel
	DDR2 AMP
	EGFR AMP
	ETV1 AMP
	FANCA DeepDel
	PTPRD DeepDel
	RAC1 AMP
	RICTOR AMP
	NTRK1 AMP
	TERT AMP
	CARD11 AMP

	FOXO1 DeepDel
	ARID5B DeepDel
	RECQL4 AMP
	CDK8 DeepDel
	CRLF2 DeepDel
	FLT1 DeepDel
	FLT3 DeepDel
	RIT AMP
	CD274 DeepDel
	IL7R AMP
	JAK2 DeepDel
	KDM5C AMP
	SDHA AMP
	SDHC AMP
	RET DeepDel
	HIST2H3C AMP
	HIST2H3D AMP
	PDCD1 DeepDel
	PMS2 AMP
	INHBA AMP
	IKZF1 AMP
	DIS3 DeepDel
	NFKBIA AMP

	BCOR AMP
	KDM6A AMP
	RBM10 AMP
	ARAF AMP
	GATA1 AMP

Table S3. Genetic alterations of organoid-610, Related to Figure 6.

Mutations	Copy number alterations
EGFR *L858R*	TERT AMP
EGFR *T790M*	SLC25A13 AMP
	XRCC2 AMP
	NKX2-1 AMP
	ARAF AMP
	CDKN2A DeepDel
	CDKN2B DeepDel
	MTAP DeepDel
	MET Loss
	BRAF Loss