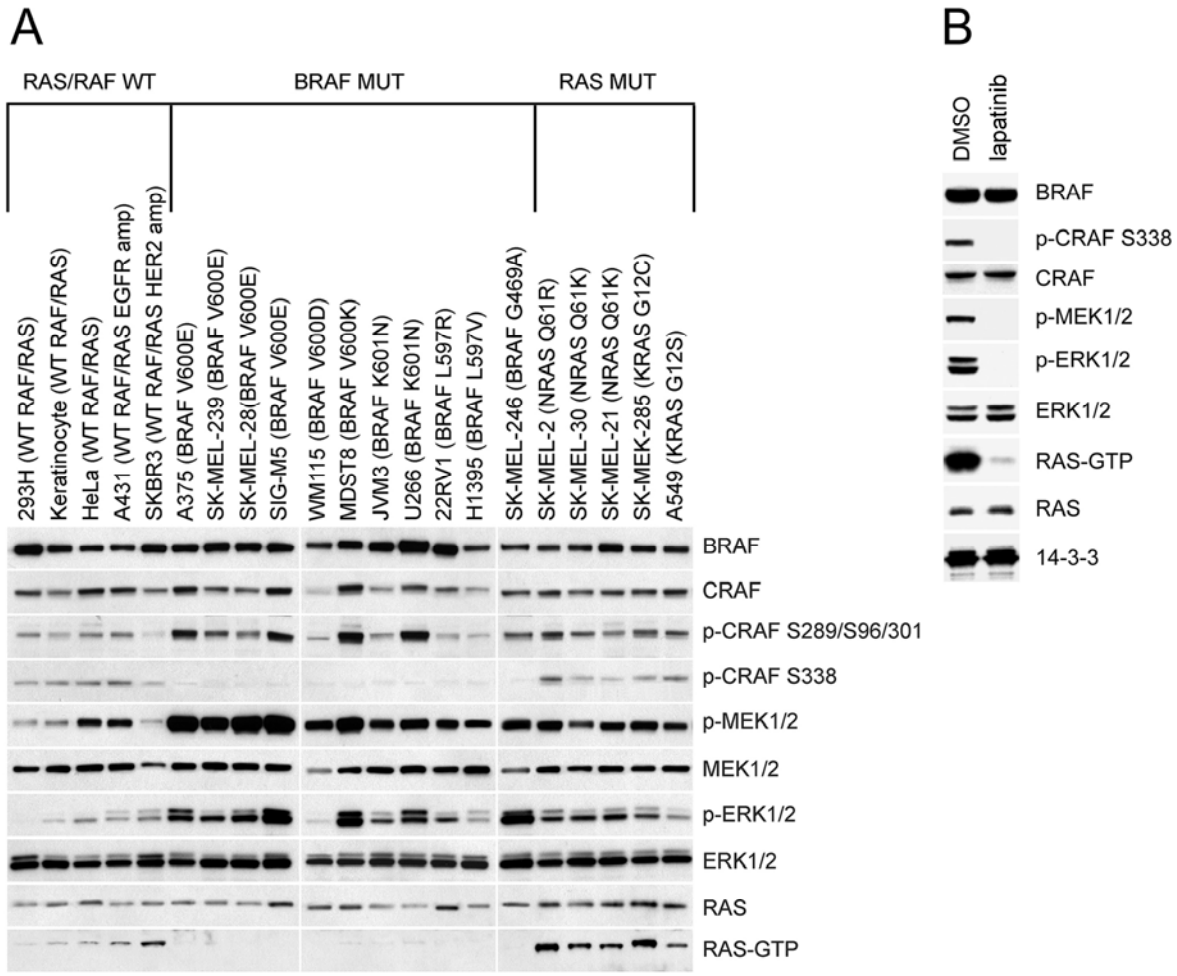
Supplemental Data



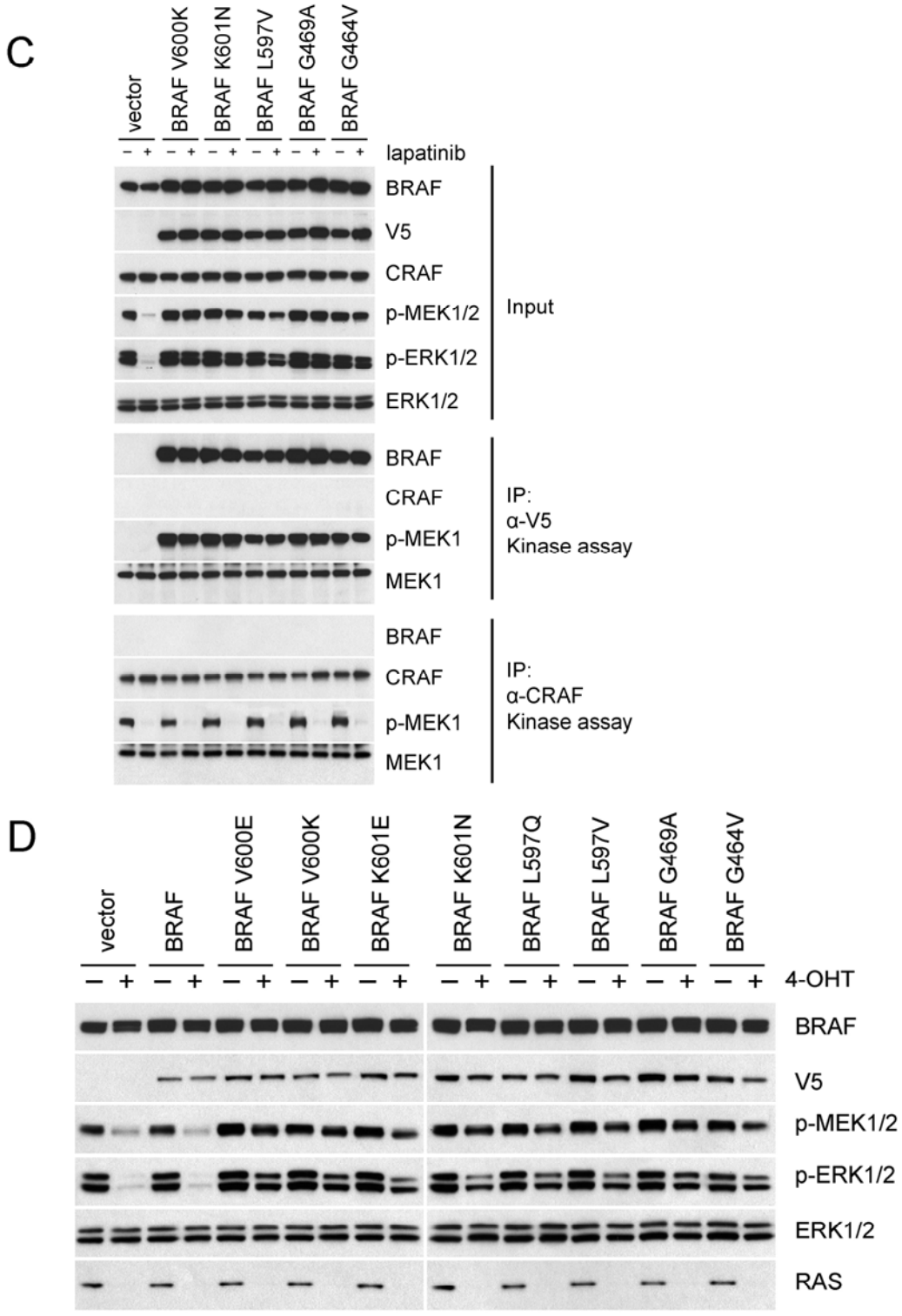

C
vector
BRAF V600K
BRAF K601N
BRAF L597V
BRAF G469A
BRAF G464V
– + – + – + – + – + – +
lapatinib
BRAF
V5
CRAF
p-MEK1/2
p-ERK1/2
ERK1/2
Input
BRAF
CRAF
p-MEK1
MEK1
IP:
α-V5
Kinase assay
BRAF
CRAF
p-MEK1
MEK1
IP:
α-CRAF
Kinase assay
D
vector
BRAF
BRAF V600E
BRAF V600K
BRAF K601E
BRAF K601N
BRAF L597Q
BRAF L597V
BRAF G469A
BRAF G464V
– + – + – + – + – + – + – + – + – + – + – +
4-OHT
BRAF
V5
p-MEK1/2
p-ERK1/2
ERK1/2
RAS

Figure S1, related to Figure 1. Activating BRAF mutants feedback inhibit cellular RAS-GTP by activating ERK signaling in a RAS-independent manner

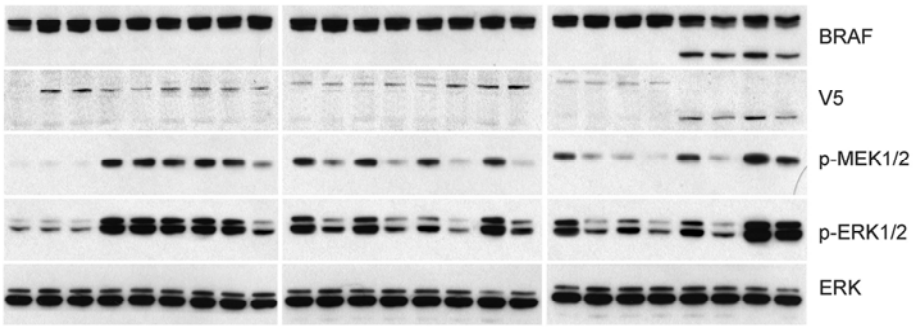
(A) Western blot analysis for components of the RAS/RAF/ERK signaling pathway in keratinocytes and in a panel of cancer cell lines harboring the indicated mutations. Cellular RAS-GTP levels were determined by the active RAS pull-down assay.

(B) SKBR3 cells were treated with either vehicle or lapatinib (1 μM) for 1 hr. Whole cell lysates were analyzed by Western blot using the indicated antibodies. RAS-GTP levels were determined using the active RAS pull-down assay.

(C) SKBR3 cells expressing V5 tagged BRAF mutants were treated with lapatinib (1 μM) or DMSO for 1 hr. Cell lysates were divided into three portions and analyzed as follows: 5% of the cell extracts were used for immunoblot of whole cell lysate (Input panels). The remainder was divided equally and then subjected to immunoprecipitation with an anti-V5 antibody or immunoprecipitation with an anti-CRAF antibody. The immunoprecipitate was then used for an in vitro kinase assay with 0.5 μg K97R MEK1 protein.

(D) Mouse embryonic fibroblasts derived from *Hras*$^{-/-}$; *Nras*$^{-/-}$; *Kras*$^{lox/lox}$; RERT $^{ert/ert}$ mice (K-Ras lox MEFs) were grown in medium with or without 1 μM 4-OHT for a week to generate isogenic cells expressing or lacking K-Ras. Cells were then transfected with pcDNA3 plasmids expressing wild-type BRAF or the BRAF mutants listed. Cells were collected 16 hr after transfection, whole cell lysates prepared and ERK pathway activity examined by Western blot.

A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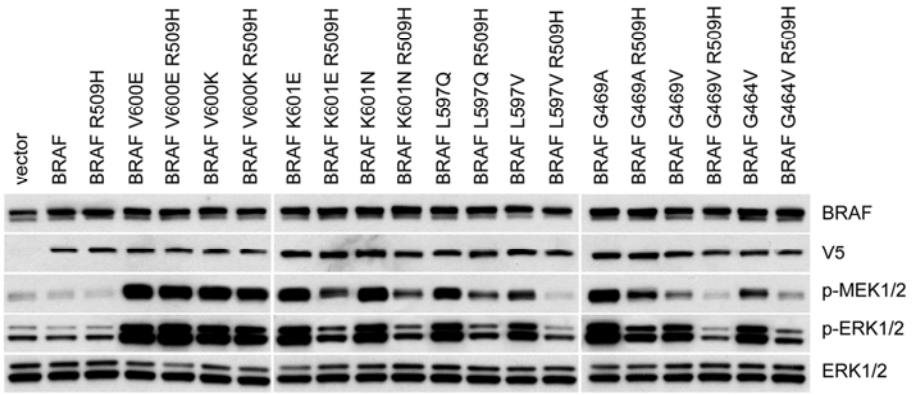
BRAF
BRAF R509H
BRAF V600E
BRAF V600E R509H
BRAF V600K
BRAF V600K R509H
BRAF K601E
BRAF K601E R509H
BRAF K601N
BRAF K601N R509H
BRAF L597Q
BRAF L597Q R509H
BRAF L597V
BRAF L597V R509H
BRAF G469A
BRAF G469A R509H
BRAF G469V
BRAF G469V R509H
BRAF G464V
BRAF G464V R509H
p61 WT
p61 WT R509H
p61 VE
p61 VE R509H
BRAF
V5
p-MEK1/2
p-ERK1/2
ERK

B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BRAF R509H
BRAF V600E
BRAF V600E R509H
BRAF V600K
BRAF V600K R509H
BRAF K601E
BRAF K601E R509H
BRAF K601N
BRAF K601N R509H
BRAF L597Q
BRAF L597Q R509H
BRAF L597V
BRAF L597V R509H
BRAF G469A
BRAF G469A R509H
BRAF G469V
BRAF G469V R509H
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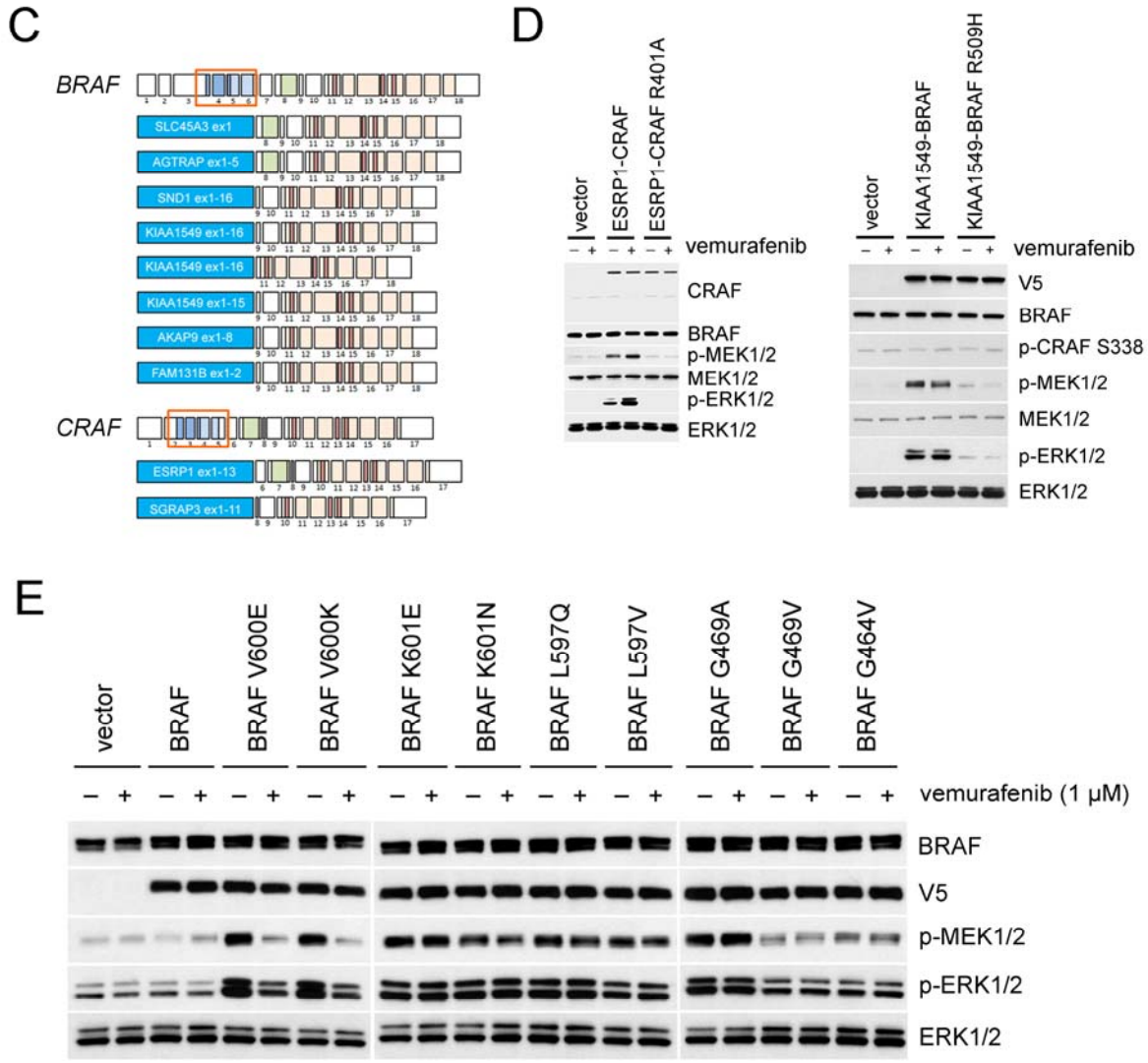
BRAF
V5
p-MEK1/2
p-ERK1/2
ERK1/2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BRAF
SLC45A3 ex1
AGTRAP ex1-5
SND1 ex1-16
KIAA1549 ex1-16
KIAA1549 ex1-16
KIAA1549 ex1-15
AKAP9 ex1-8
FAM131B ex1-2
CRAF
ESRP1 ex1-13
SGRAP3 ex1-11
D
vector
ESRP1-CRAF
ESRP1-CRAF R401A
vemurafenib
CRAF
BRAF
p-MEK1/2
MEK1/2
p-ERK1/2
ERK1/2
vector
KIAA1549-BRAF
KIAA1549-BRAF R509H
vemurafenib
V5
BRAF
p-CRAF S338
p-MEK1/2
MEK1/2
p-ERK1/2
ERK1/2
E
vector
BRAF
BRAF V600E
BRAF V600K
BRAF K601E
BRAF K601N
BRAF L597Q
BRAF L597V
BRAF G469A
BRAF G469V
BRAF G464V
vemurafenib (1 μM)
BRAF
V5
p-MEK1/2
p-ERK1/2
ERK1/2

Figure S2, related to Figure 2. ERK signaling driven by oncogenic RAF dimers is insensitive to vemurafenib treatment

(A) K-Ras lox MEFs were grown in medium with 1 μM 4-OHT for a week. Cells were then transfected with pcDNA3 plasmids encoding wild-type BRAF or the BRAF mutants listed. Whole cell lysates were prepared 16 hr after transfection. ERK signaling was examined by Western blot.

(B) *Raf1* knockout MEFs were transfected with pcDNA3 vector or pcDNA3 plasmids expressing the listed BRAF mutants. 24 hr after transfection, cells were collected and lysed. Cell lysates were assayed by Western blot using the antibodies indicated.

(C) Schematic representation of RAF fusions identified in human cancers. In all cases, the RAS-binding domain is deleted from the resulting fusion.

(D) SKBR3 cells were transfected with pcDNA3 vector, pcDNA3-ESRP1-CRAF-FLAG, pcDNA3-ESRP1-CRAF R401A-FLAG, KIAA1549-BRAF-V5, or KIAA1549-BRAF R509H-V5. 24 hr after transfection, cells were treated with either vehicle or vemurafenib (1 μM, 1 hr) followed by Western blot anlaysis using the indicated antibodies.

(E) *Raf1* knockout MEFs were transfected with pcDNA3 vector or pcDNA3 plasmids expressing the listed BRAF proteins. 24 hr after transfection, cells were treated with either vehicle or vemurafenib (1 μM for 1 hr). Immunoblotted was then performed using the antibodies indicated.

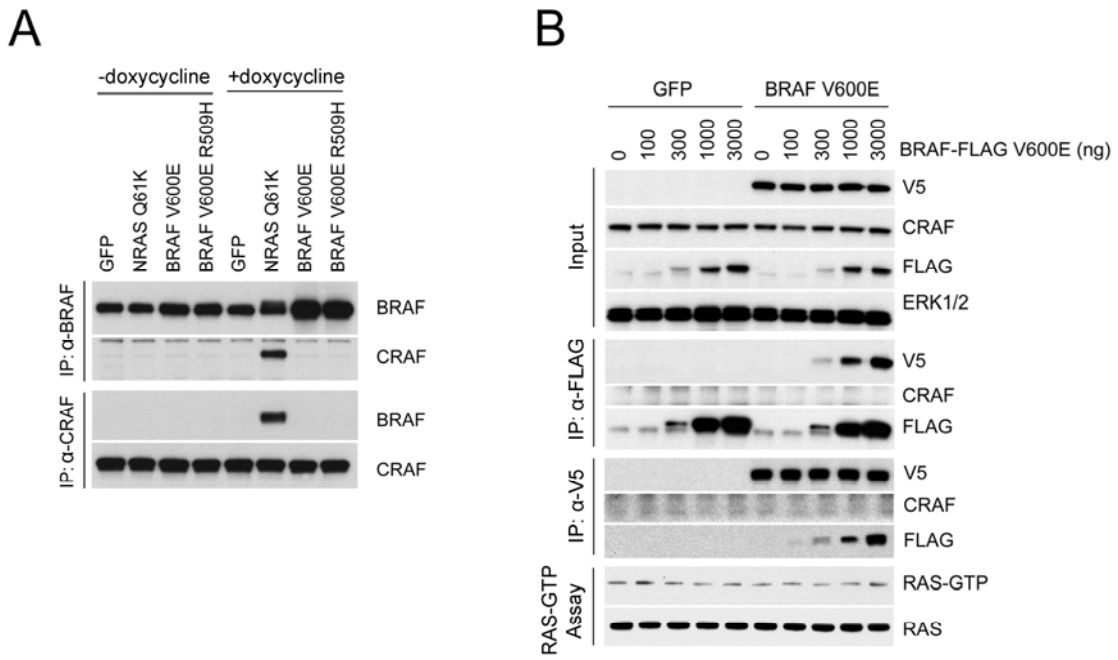
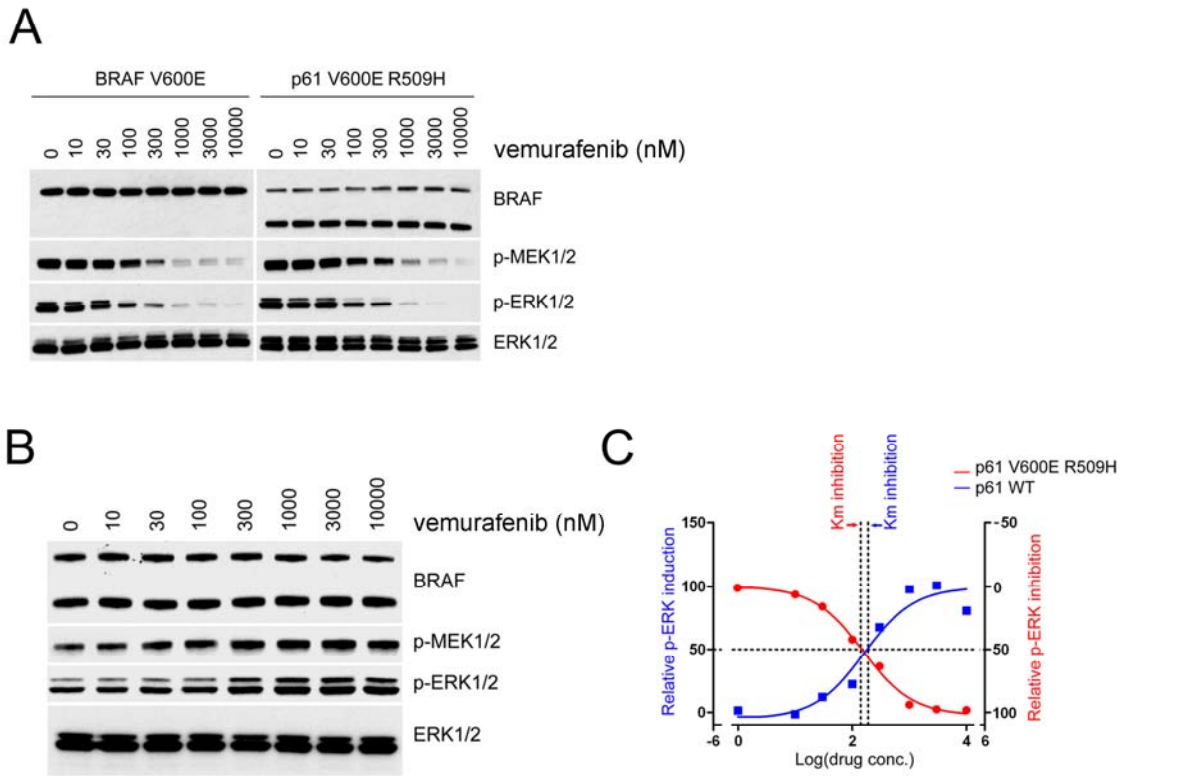


Figure S3, related to Figure 3. BRAF V600E homo-dimers are induced by BRAF V600E overexpression

(A) A375 clones expressing inducible GFP, NRAS Q61K, BRAF V600E or BRAF V600E R509H were treated with 0.5 μg/ml doxycycline for 24 hr. Cell lysates were prepared from the indicated cells and subjected to immunoprecipitation using either anti-BRAF or anti-CRAF antibodies. The isolated protein complexes were then eluted and assayed by Western blot with anti-BRAF or anti-CRAF antibodies as indicated.

(B) A375 cells expressing either inducible GFP or V5-tagged BRAF V600E were cultured in doxycycline containing medium for 1 day. The cells were then transfected with the indicated amounts of pcDNA3-BRAF V600E-FLAG plasmid. The BRAF V600E dimer was isolated by immunoprecipitation and assayed by Western blot using the indicated antibodies. The levels of RAS-GTP were determined using the active RAS pull-down assay.


A
BRAF V600E
p61 V600E R509H
0 10 30 100 300 1000 3000 10000
vemurafenib (nM)
BRAF
p-MEK1/2
p-ERK1/2
ERK1/2
B
0 10 30 100 300 1000 3000 10000
vemurafenib (nM)
BRAF
p-MEK1/2
p-ERK1/2
ERK1/2
C
Km inhibition
Km inhibition
p61 V600E R509H
p61 WT
Relative p-ERK induction
Relative p-ERK inhibition
150
100
50
0
-50
0
50
100
-6 0 2 4 6
Log(drug conc.)
D
SK-MEL-239
SK-MEL-239 C4
0 10 30 100 300 1000 3000 10^4
dabrafenib (nM)
BRAF
p-MEK
p-ERK
SK-MEL-239
SK-MEL-239 C4
0 10 30 100 300 1000 3000 10^4
LGX818 (nM)
BRAF /CRAF
p-MEK
p-ERK
SK-MEL-239
SK-MEL-239 C4
0 1 3 10 30 100 300 1000 3000
BGB659 (nM)
BRAF /CRAF
p-MEK
p-ERK
SK-MEL-239
SK-MEL-239 C4
0 1 3 10 30 100 300 1000 3000
BGB283 (nM)
BRAF /CRAF
p-MEK
p-ERK
SK-MEL-239
SK-MEL-239 C4
0 10 30 100 300 1000 3000 10^4
PLX7904 (nM)
BRAF
p-MEK
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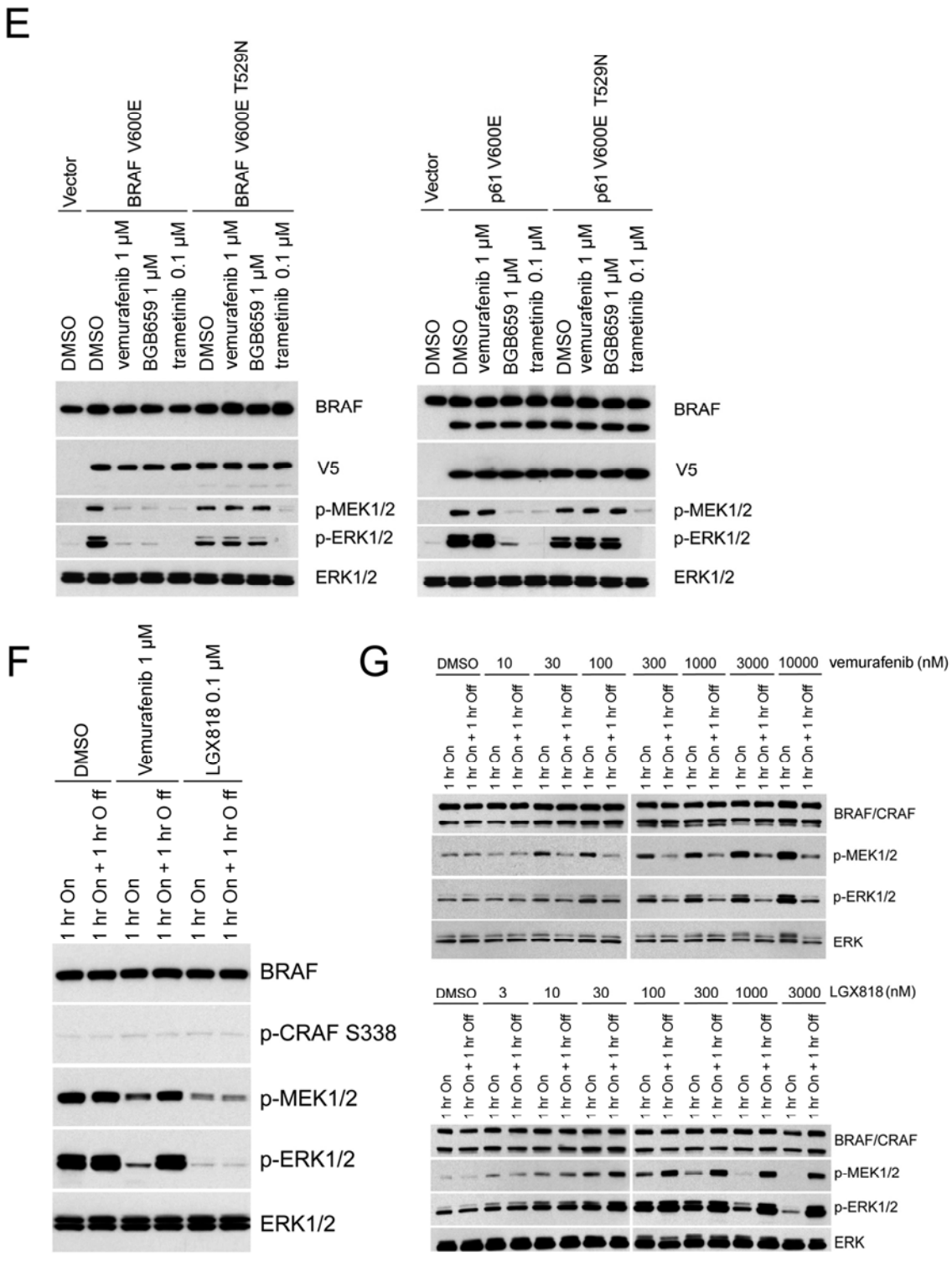
PLX8394 (nM)
BRAF
p-MEK
p-ERK


E
Vector
BRAF V600E
BRAF V600E T529N
DMSO
DMSO
vemurafenib 1 μM
BGB659 1 μM
trametinib 0.1 μM
DMSO
vemurafenib 1 μM
BGB659 1 μM
trametinib 0.1 μM
BRAF
V5
p-MEK1/2
p-ERK1/2
ERK1/2
Vector
p61 V600E
p61 V600E T529N
DMSO
DMSO
vemurafenib 1 μM
BGB659 1 μM
trametinib 0.1 μM
DMSO
vemurafenib 1 μM
BGB659 1 μM
trametinib 0.1 μM
BRAF
V5
p-MEK1/2
p-ERK1/2
ERK1/2
F
DMSO
Vemurafenib 1 μM
LGX818 0.1 μM
1 hr On
1 hr On + 1 hr O ff
1 hr On
1 hr On + 1 hr O ff
1 hr On
1 hr On + 1 hr O ff
BRAF
p-CRAF S338
p-MEK1/2
p-ERK1/2
ERK1/2
G
DMSO
10
30
100
300
1000
3000
10000
vemurafenib (nM)
1 hr On
1 hr On + 1 hr Off
BRAF/CRAF
p-MEK1/2
p-ERK1/2
ERK
DMSO
3
10
30
100
300
1000
3000
LGX818 (nM)
1 hr On
1 hr On + 1 hr Off
BRAF/CRAF
p-MEK1/2
p-ERK1/2
ERK

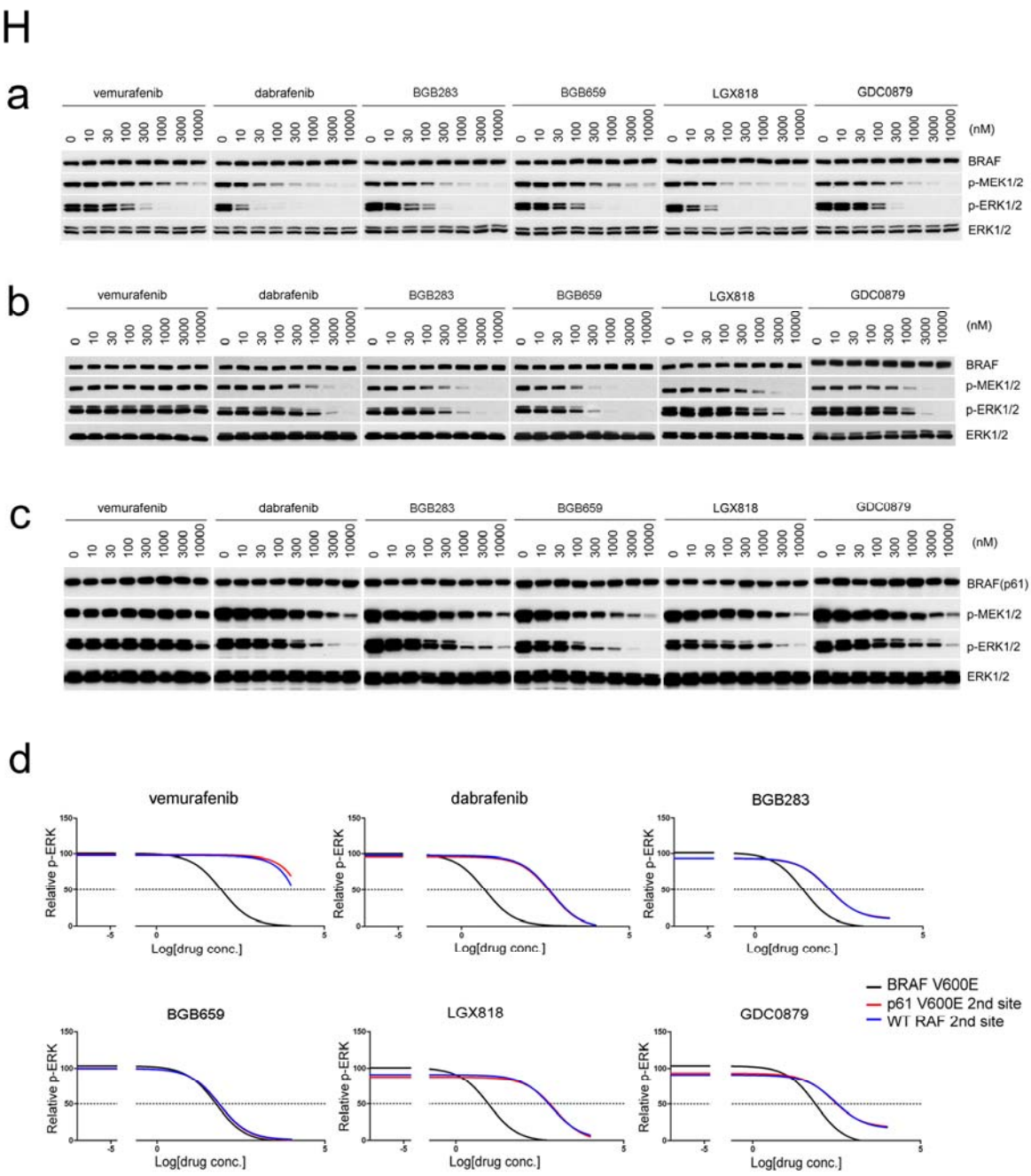


Figure S4, related to Figure 4. Binding to one protomer of RAF dimers reduces the drug affinity to the other protomer

(A) BRAF V600E and p61 V600E R509H were transiently expressed in SKBR3 cells. After 24 hr, the cells were treated with 1 μM lapatinib for 1 hr and then vemurafenib for 1 hour at the indicated doses. The cell lysates were then assayed by Western blot using the antibodies indicated.

(B) p61 WT BRAF was transiently expressed in SKBR3 cells. After 24 hr, cells were treated with lapatinib (1 μM for 1 hr) followed by vemurafenib for 1 hr at the concentrations indicated. The expression of p61 WT and activation of ERK signaling were determined by Western blot.

(C) Vemurafenib mediated inhibition of p61 V600E R509H (as shown in (A)) and activation of p61 WT (as shown in (B)) are shown as relative changes in p-ERK levels as quantitated by densitometry analysis as a function of drug concentration. Response curves were generated using Prism6.

(D) SK-MEL-239 parental or C4 sub-clone cells were treated with increasing concentrations of the indicated RAF inhibitors for 1 hr. ERK pathway activity was then determined by Western blot using the antibodies indicated.

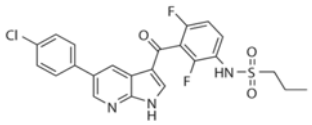
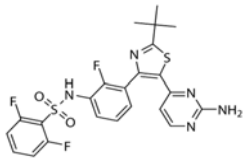
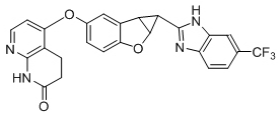
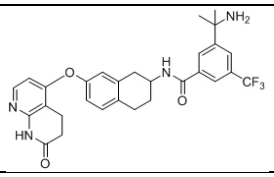
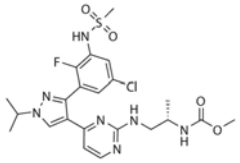
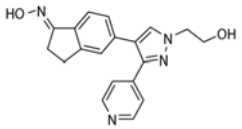
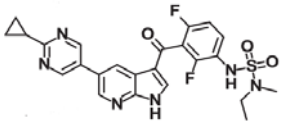
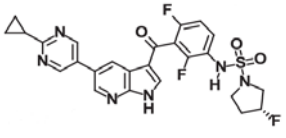
(E) SKBR3 cells were transfected with empty vector, or plasmids expressing the indicated BRAF mutants. 24 hr after transfection, cells were treated with the indicated compounds for 1 hr. Samples were then analyzed by Western blot using the antibodies indicated.

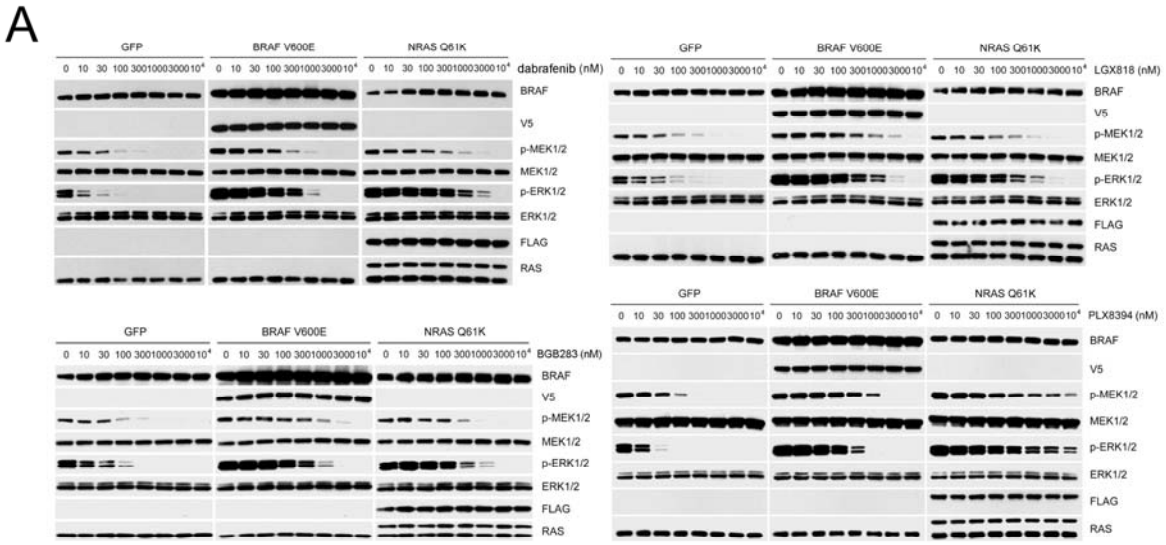
(F) A375 cells were treated with DMSO or the compounds indicated for 1 hr. Cells were then washed with pre-warmed drug free media three times and then incubated in drug-free media for another hour. Cells were then lysed and examined by Western blot using the antibodies indicated.

(G) SK-MEL-30 cells were treated with vemurafenib or LGX818 at the concentration indicated for 1 hr followed by drug washout as described above and then incubation in drug-free medium for another hour. Cells were then collected and lysed. Western blot analysis was then performed using the antibodies indicated.

(H) A375 cells (a) were treated with the indicated compounds at the listed concentrations for 1 hr. SK-MEL-30 (b) and SK-MEL-239 C4 (c) cells were treated with 1 μM LGX818 for 1 hr, following by drug washout, followed by treatment with the indicated compounds at the concentrations listed for an additional hour. Lysates were then analyzed by Western blot using the antibodies indicated. p-ERK levels were quatitated by densitometry analysis. The data collected at all doses were then analyzed using Prism6 to generate the dose-dependent inhibition curves shown in (d).

Table S1, related to Figure 4. Chemical structures of the RAF inhibitors

Compound Name	Structure	Type
vemurafenib		Type I
dabrafenib		Type I
BGB283		Type II
BGB659		Type II
LGX818		Type I
GDC0879		Type I
PLX7904		Type I
PLX8394		Type I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BRAF V600E
NRAS Q61K
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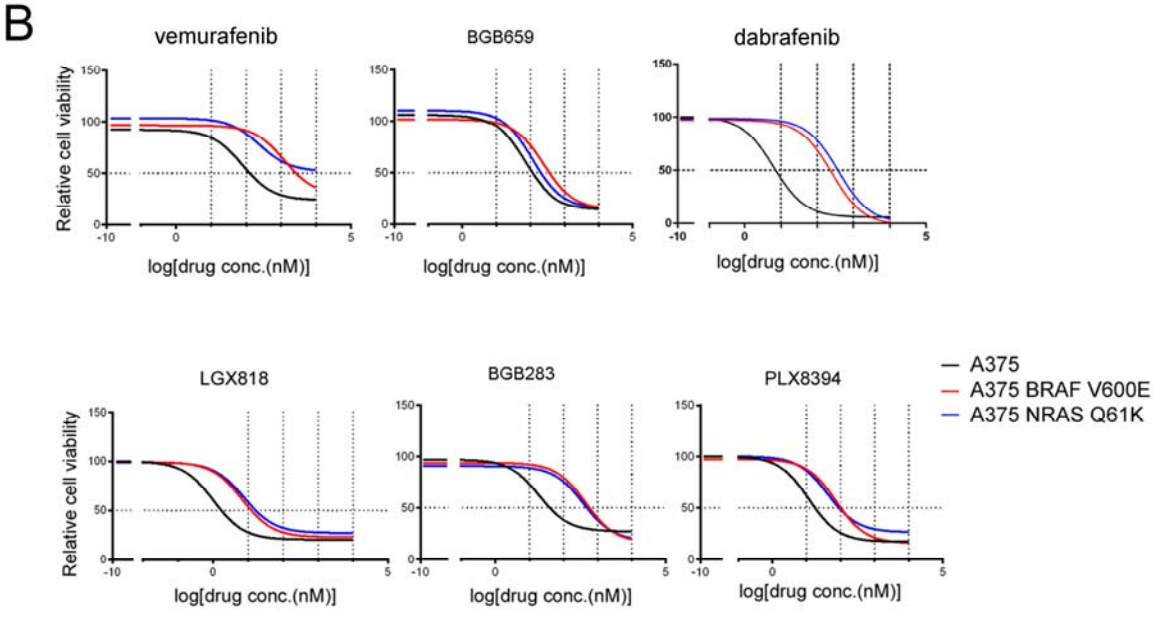
dabrafenib (nM)
LGX818 (nM)
BGB283 (nM)
PLX8394 (nM)
BRAF
V5
p-MEK1/2
MEK1/2
p-ERK1/2
ERK1/2
FLAG
RAS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vemurafenib
BGB659
dabrafenib
LGX818
BGB283
PLX8394
Relative cell viability
log[drug conc.(nM)]
A375
A375 BRAF V600E
A375 NRAS Q61K

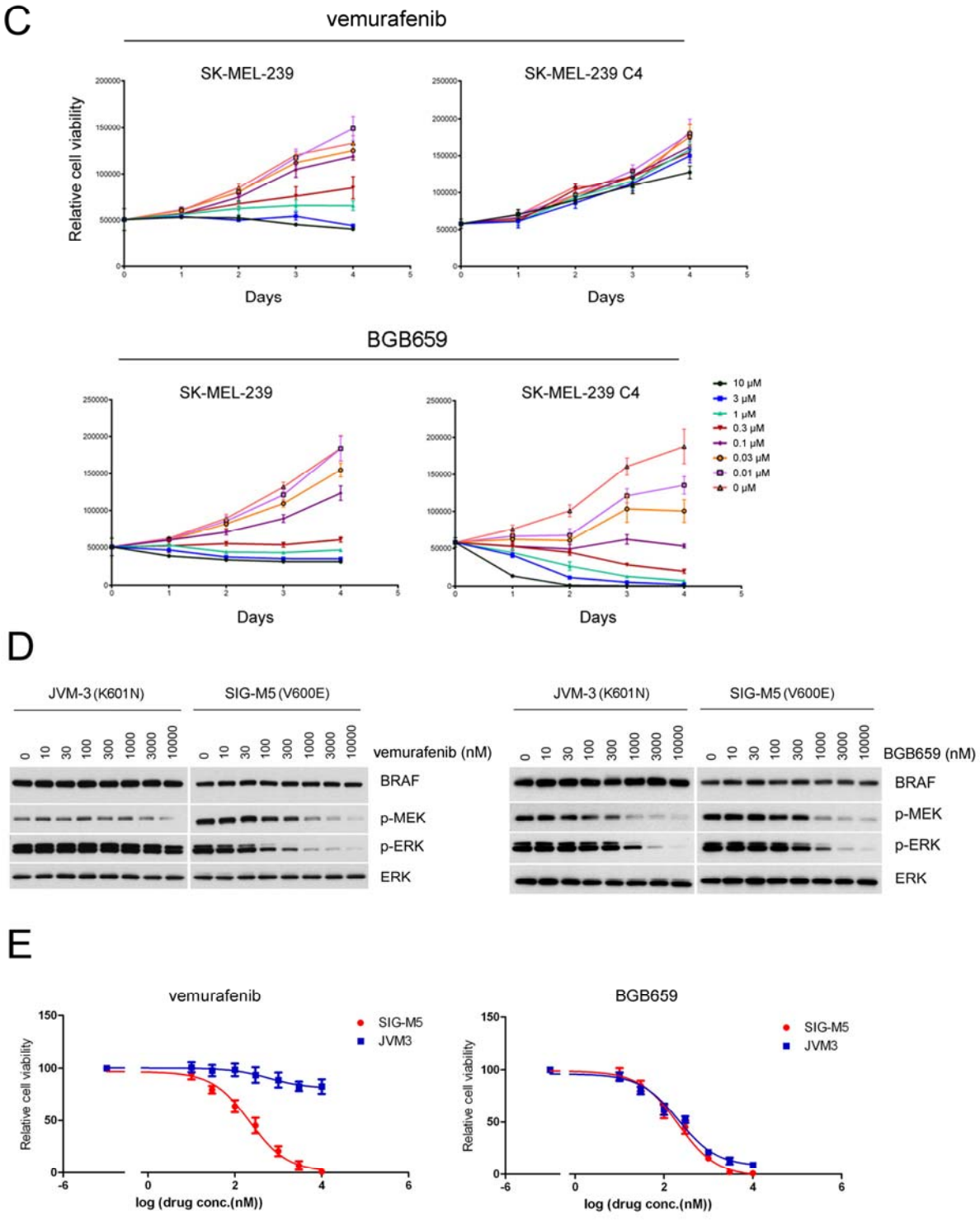

C
vemurafenib
SK-MEL-239
SK-MEL-239 C4
Relative cell viability
Days
BGB659
10 μM
3 μM
1 μM
0.3 μM
0.1 μM
0.03 μM
0.01 μM
0 μM
D
JVM-3 (K601N)
SIG-M5 (V600E)
vemurafenib (nM)
BGB659 (nM)
0
10
30
100
300
1000
3000
10000
BRAF
p-MEK
p-ERK
ERK
E
vemurafenib
BGB659
SIG-M5
JVM3
Relative cell viability
log (drug conc.(nM))

Figure S5, related to Figure 5. BGB659 inhibits tumor cells driven by mutant BRAF dimers and the BRAF V600E monomer at similar doses.

(A) A375 cells expressing doxycycline inducible GFP, BRAF V600E or NRAS Q61K were treated with doxycycline (2 μg/ml, 24 hr) and then treated with the compounds indicated for 1 hr. Cell lysates were then analyzed by Western blot using the antibodies indicated.

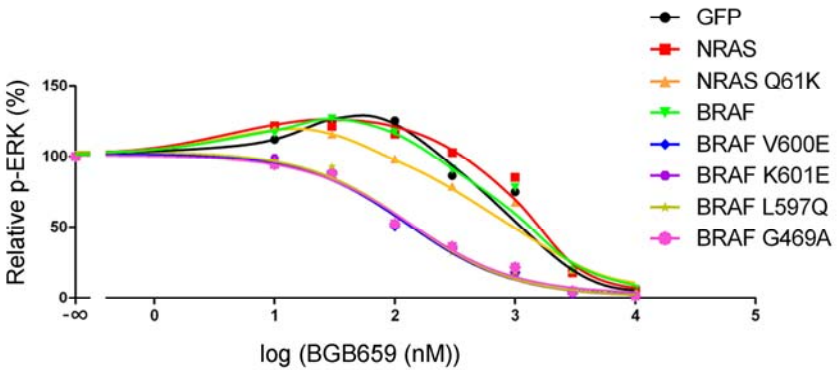
(B) Isogenic A375 cells as described in (A) were cultured in the medium containing 2 μg/ml doxycycline for 1 day. Cells (1000 cells/well) were then incubated with the compounds indicated at concentrations of 0, 10, 30, 100, 300, 1000, 3000, 10000 nM for 3 days. The effects of drug on cell growth were quantitated using the ATP-Glo assay on day 3. Graphs were generated using Prism6 based on the average of 8 replicates.

(C) SK-MEL-239 and SKMEL-239 C4 cells were grown in increasing concentrations of vemurafenib or BGB659 and then the effects of each compound on cell growth was quantitated using the ATP-Glo assay. Cells were initially seeded in 96 well plates at 1,000 cells/well. (n=8, error bars indicated S.D.)

(D) JVM-3 (BRAF K601E) and SIG-M5 (BRAF V600E) cells were treated with increasing concentrations of vemurafenib or BGB659 for 1 hour. Cell lysates were then prepared and analyzed by Western blot using the antibodies indicated.

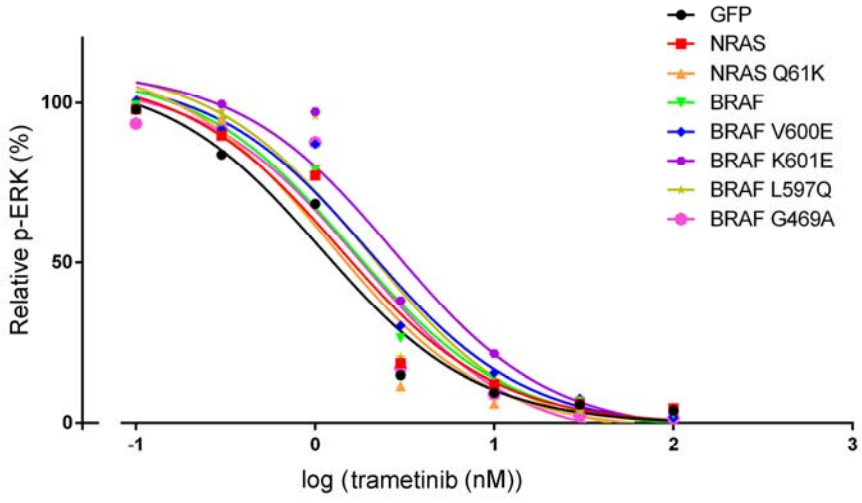
(E) JVM-3 (BRAF K601E) and SIG-M5 (BRAF V600E) cells were treated with increasing concentrations of vemurafenib or BGB659 and cell growth was quantitated using the ATP-Glo assay after 3 days of treatment. Cells were initially seeded in 96 well plates at 5,000 cells/well. (n=8, error bars indicated S.D.)

A

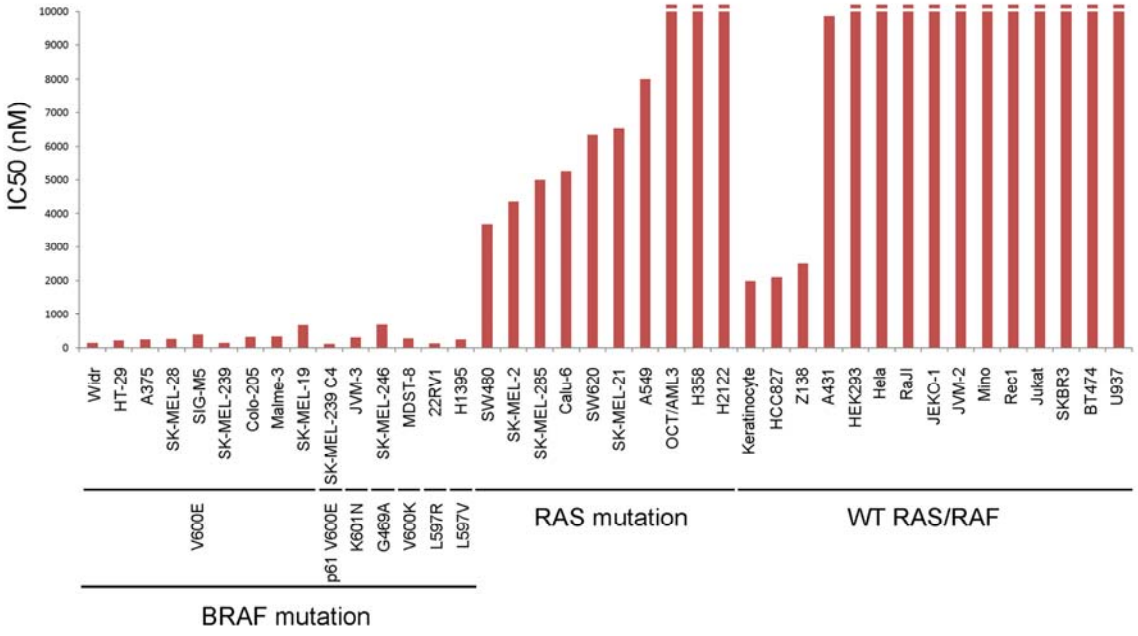


p-ERK Inhibition	GFP	NRAS	NRAS Q61K	BRAF	BRAF V600E	BRAF K601E	BRAF L597Q	BRAF G469A
IC_{50} (nM)	1621.8	2041.738	1513.56	1698.24	138.0384	147.9108	144.544	141.2538
IC_{70} (nM)	3162.27	3630.781	2630.268	3162.27	323.5937	354.8134	346.7369	354.8134
IC_{90} (nM)	6918.31	6456.542	7079.458	6606.93	1318.257	1412.538	1513.561	1584.893

B



C


IC50 (nM)
10000
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
Widr
HT-29
A375
SK-MEL-28
SIG-M5
SK-MEL-239
Colo-205
Malme-3
SK-MEL-19
SK-MEL-239 C4
JVM-3
SK-MEL-246
MDST-8
22RV1
H1395
SW480
SK-MEL-2
SK-MEL-285
Calu-6
SW620
SK-MEL-21
A549
OCT/AML3
H358
H2122
Keratinocyte
HCC827
Z138
A431
HEK293
Hela
Raji
JEKC-1
JVM-2
Mino
Rec1
Jukat
SKBR3
BT474
U937
V600E
p61 V600E
K601N
G469A
V600K
L597R
L597V
RAS mutation
WT RAS/RAF
BRAF mutation

D

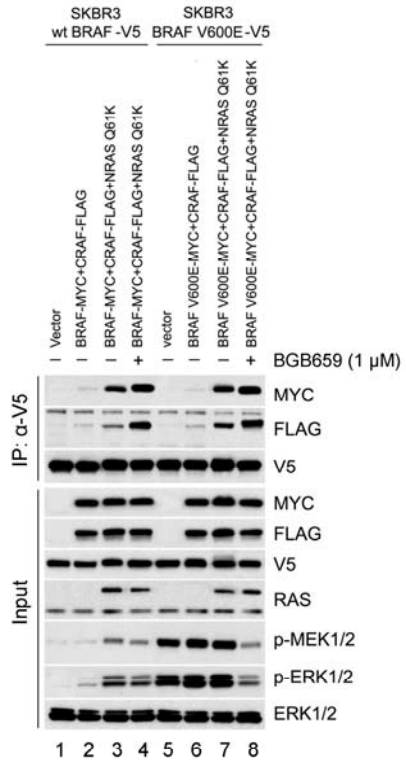

SKBR3 wt BRAF -V5
SKBR3 BRAF V600E-V5
Vector
BRAF-MYC+CRAF-FLAG
BRAF-MYC+CRAF-FLAG+NRAS Q61K
BRAF-MYC+CRAF-FLAG+NRAS Q61K
vector
BRAF V600E-MYC+CRAF-FLAG
BRAF V600E-MYC+CRAF-FLAG+NRAS Q61K
BRAF V600E-MYC+CRAF-FLAG+NRAS Q61K
BGB659 (1 μM)
IP: α-V5
MYC
FLAG
V5
Input
MYC
FLAG
V5
RAS
p-MEK1/2
p-ERK1/2
ERK1/2
1 2 3 4 5 6 7 8

Figure S6, related to Figure 6. RAS-dependent RAF kinase activity is less sensitive to BGB659 because the drug induces RAS-dependent RAF dimerization

(A, B) BRAF, BRAF mutants tagged with V5, NRAS and NRAS Q61K tagged with FLAG were expressed in NIH3T3 cells upon cultured in media containing doxycycline (30 ng/ml). Cells were then treated for 1 hr with increasing concentrations of BGB659 or trametinib. Whole cell lysates were assayed by Western blot and p-ERK level were quantitated by densitometry analysis. Relative p-ERK levels were normalized to the p-ERK level of untreated cells expressing each mutant construct. The effect of BGB659 (A) and trametinib (B) on p-ERK expression was then analyzed using Prism6 to generate the IC_{50}, IC_{70}, and IC_{90} values for BGB659 shown in the table in (A) .

(C) The indicated cell lines were grown in increasing concentrations of BGB659 and IC_{50} for cell growth inhibition for BGB659 were calculated using the ATP-Glo assay. Cells were treated for 3 days with 8 different drug concentrations (0, 10, 30, 100, 300, 1,000, 3,000 and 10,000 nM) with 8 replicates at each concentration. IC50 values were generated using Prism6.

(D) SKBR3 cells stably expressing BRAF-V5 and BRAF V600E-V5 were transfected with the indicated plasmids. After 24 hr, cells were treated with lapatinib (1 μM) for 1 hr, followed by DMSO or BGB659 (1 μM) for 1 hr. Cells were then collected and lysed in 1% NP40 buffer. Immunoprecipitations were performed with anti-V5 antibody to quantitate levels of homo-BRAF-dimer and hetero-BRAF/CRAF-dimer expressoin. Samples were analyzed by Western blot using the antibodies indicated.

Supplemental Experimental Procedures

Generation of RAS-less cells

Hras$^{-/-}$; *Nras*$^{-/-}$; *Kras*$^{lox/lox}$; RERT $^{ert/ert}$ (K-Ras lox) MEFs were generously provide by Mariano Barbacid MEF cells were isolated from embryonic day 13.5 embryos and immortalized using standard methods. The protocol for the generation of Ras-less cells was followed as previously reported (Drosten et al., 2010).

Transfections

Cells were seeded in 60 mm or 100 mm plates and transfected the following day using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. A ratio of DNA to lipofectamine of 1 µg DNA/3 µl lipofectamine was employed.

Cell growth assays

Cells were seeded into 96 well plates at 1000 cells per well. Cell growth was quantitated using the ATP-Glo assay (Promega) every 24 hr. For each condition, 8 replicates of each concentration were measured. IC_{50} values were calculated using Graphpad Prizsm 6.

Gene inducible expression cell system

Retrovirus encoding rtTA or the BRAF or NRAS genes was packaged in Phoenix-AMPHO cells obtained from ATCC. The medium containing virus was filtered with 0.45 PVDF filters followed by incubation with the target cells for 6 hr. Cells were then maintained in virus free media for 2 days. Cells were selected using Puromycin (2 µg/ml) or Hygromycin (250 µg/ml) for 3 days. The positive infected cell populations were further sorted using GFP as a marker after overnight exposing to 1µg/ml doxycycline. GFP positive cells were then cultured and expanded in medium without doxycycline but with antibiotics.

Active RAS pull-down assay

Cells were cultured in 10cm dishes until 70-80% confluence. GTP-bound Ras was quantitated using the RAF1 Ras-binding domain (RBD) pull-down from Detection Kit (Thermo Scientific) or RAS activation assay BIOCHEM KIT (Cytoskeleton), as instructed by the manufacturers.

Supplemental References

Drosten, M., Dhawahir, A., Sum, E.Y., Urosevic, J., Lechuga, C.G., Esteban, L.M., Castellano, E., Guerra, C., Santos, E., and Barbacid, M. (2010). Genetic analysis of Ras signalling pathways in cell proliferation, migration and survival. The EMBO journal *29*, 1091-1104.