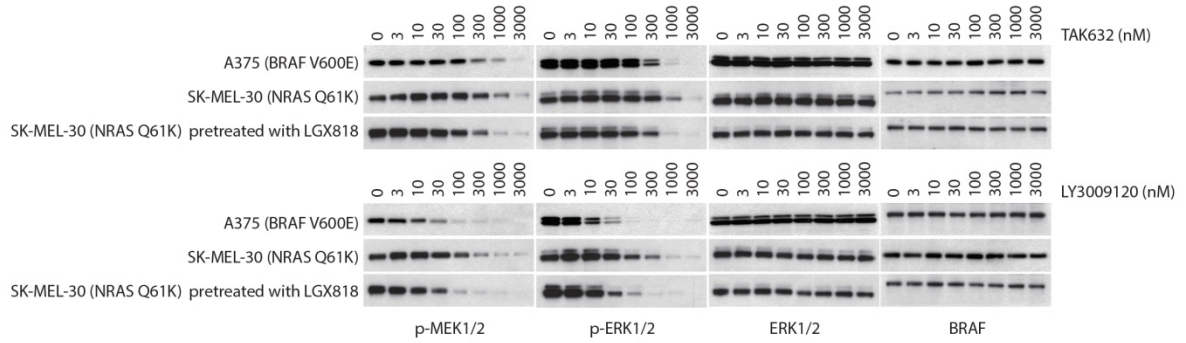
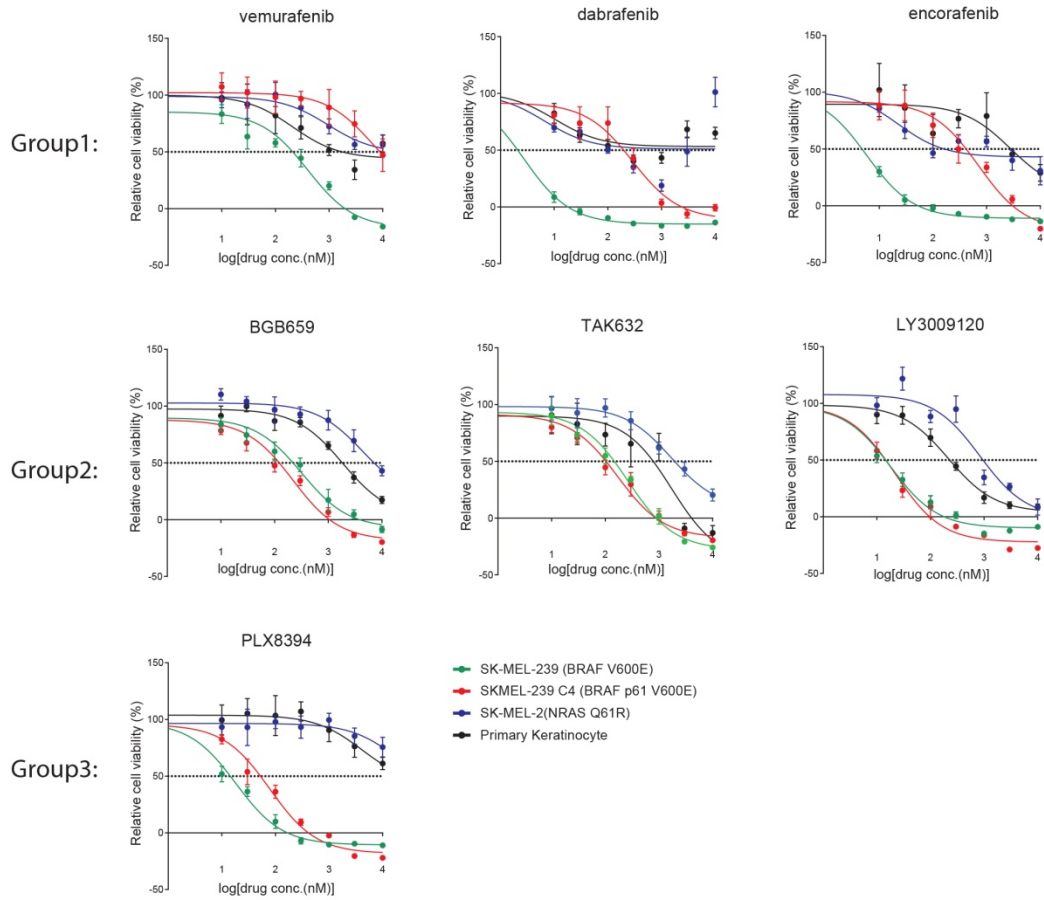


# Supplementary Figure 1

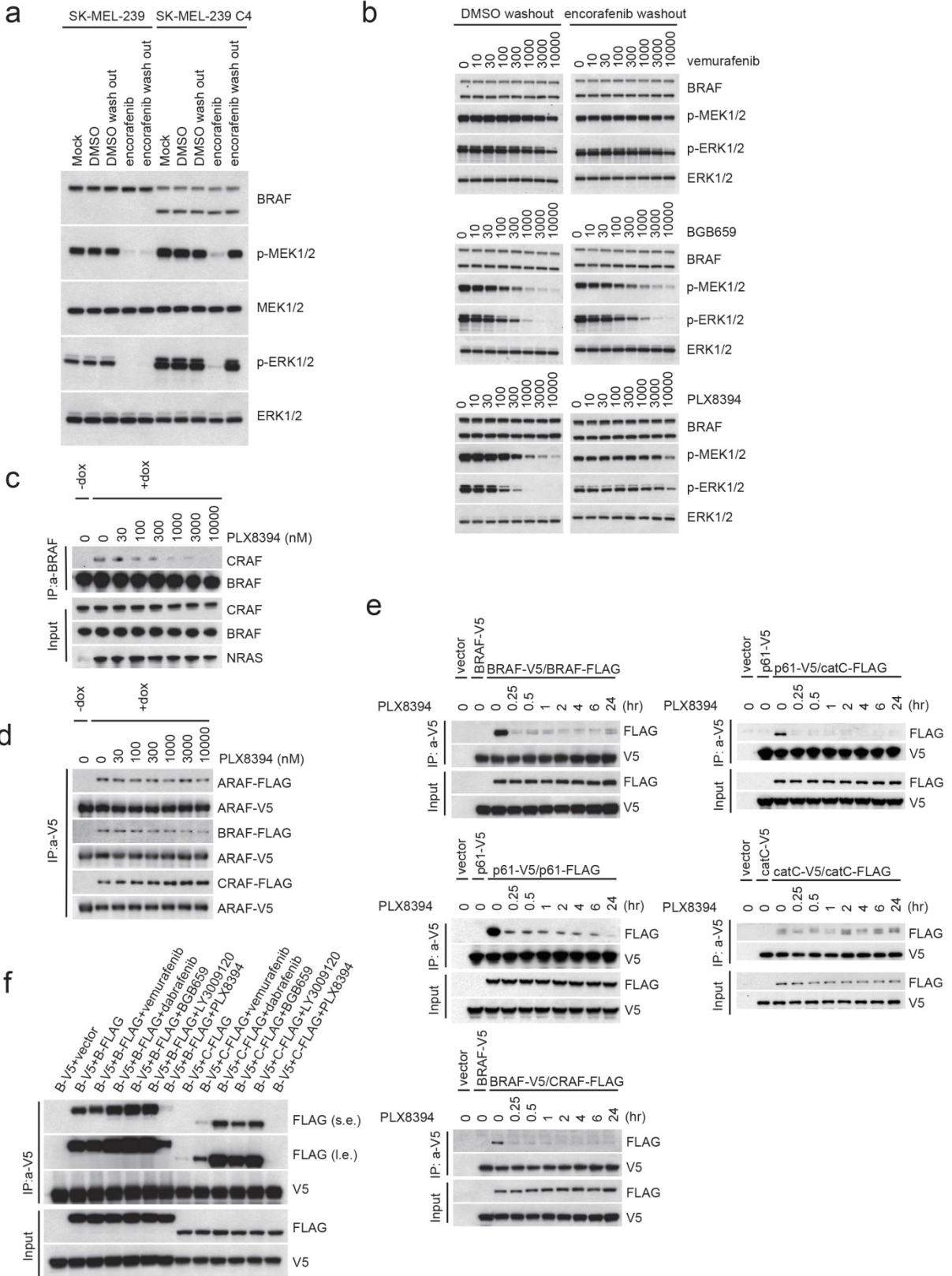
a



b

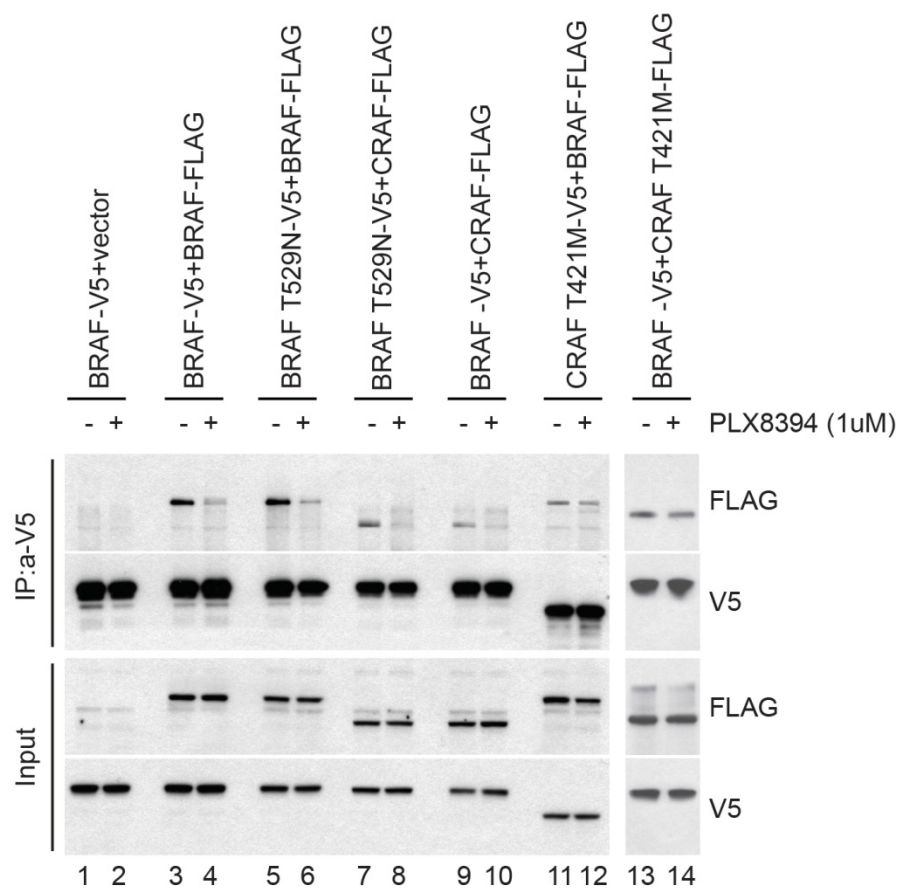


# Supplementary Figure 2

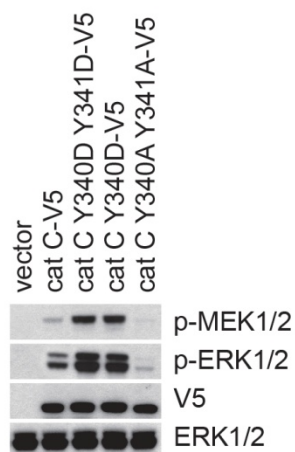


# Supplementary Figure 3

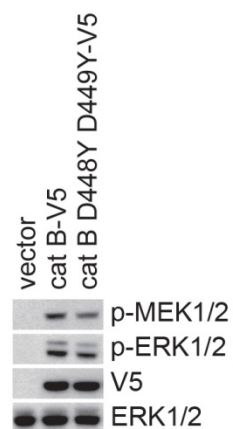
a



b

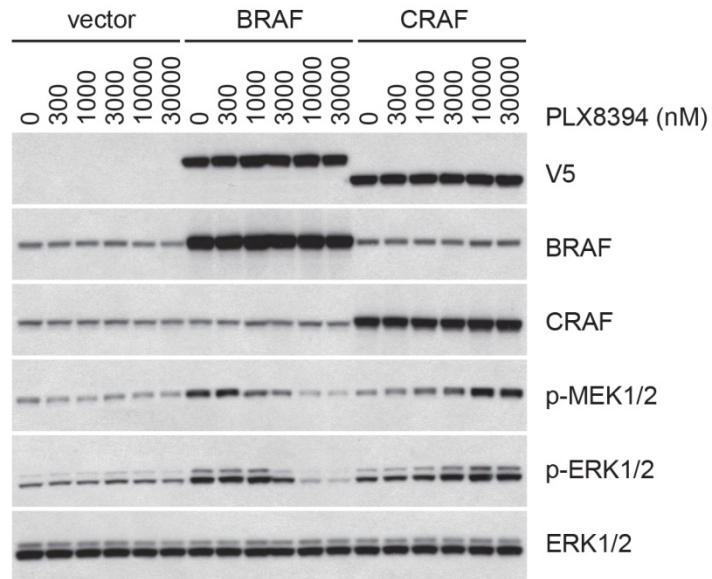


c

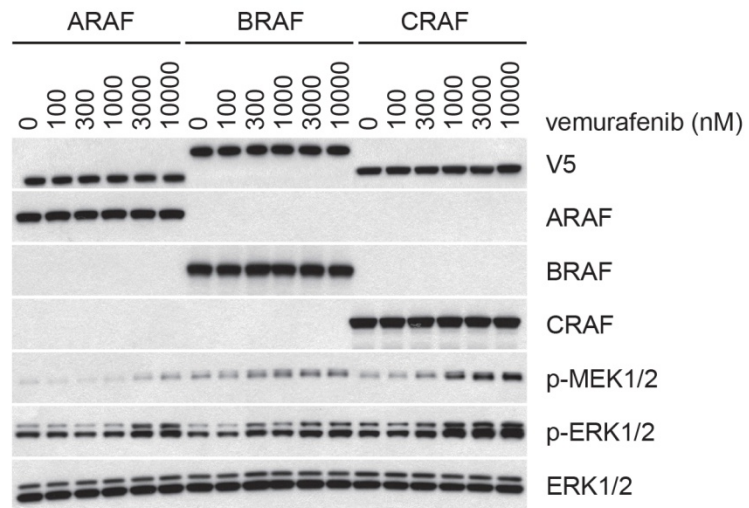


# Supplementary Figure 4

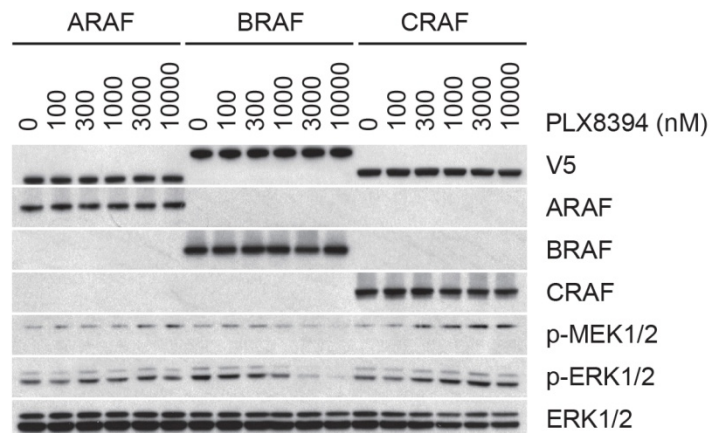
a



b

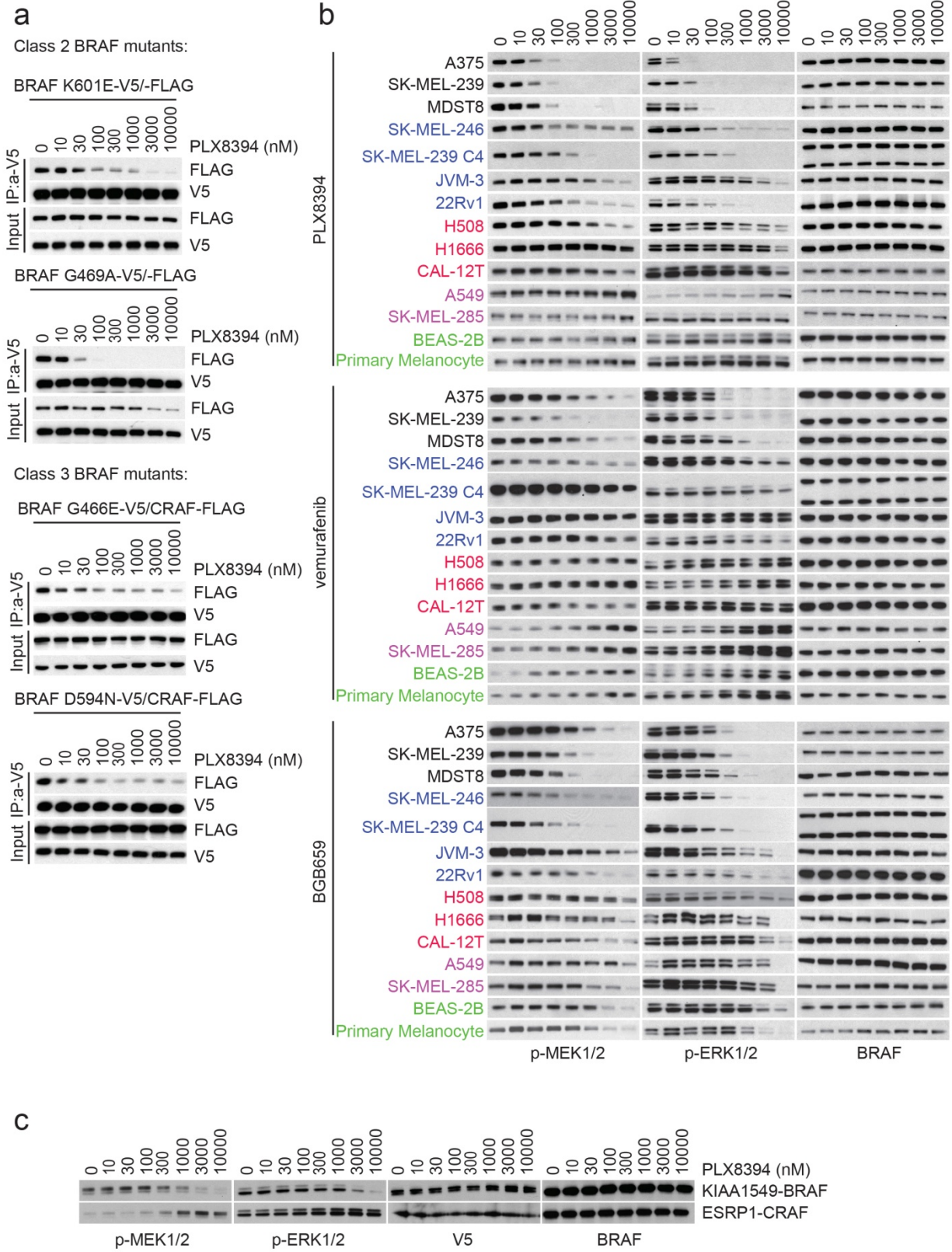


c

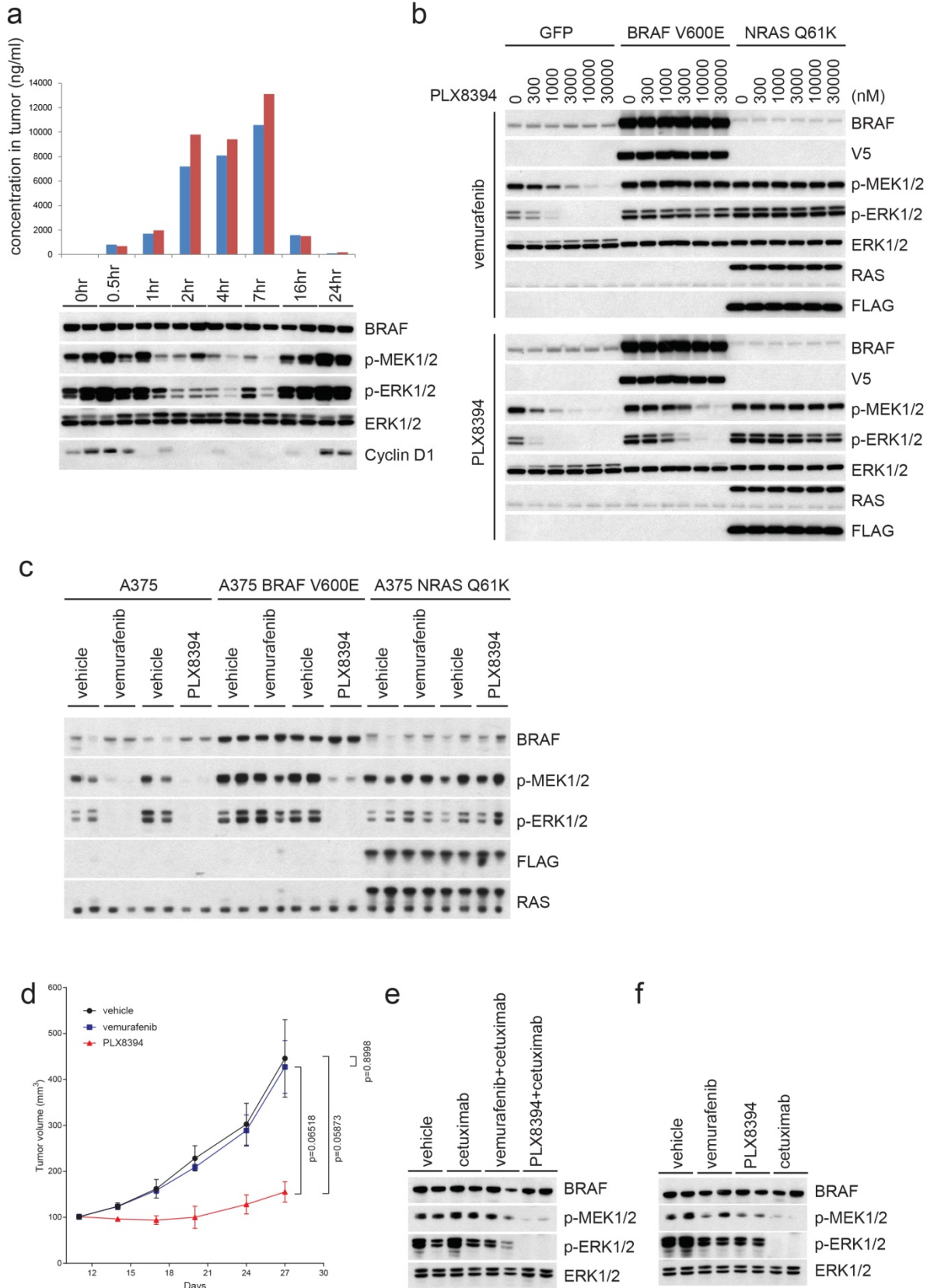




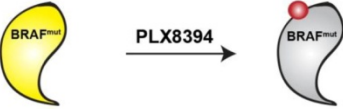
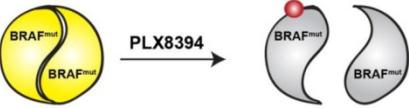
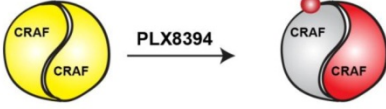
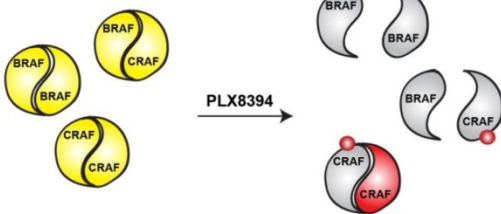
# Supplementary Figure 5



# Supplementary Figure 6

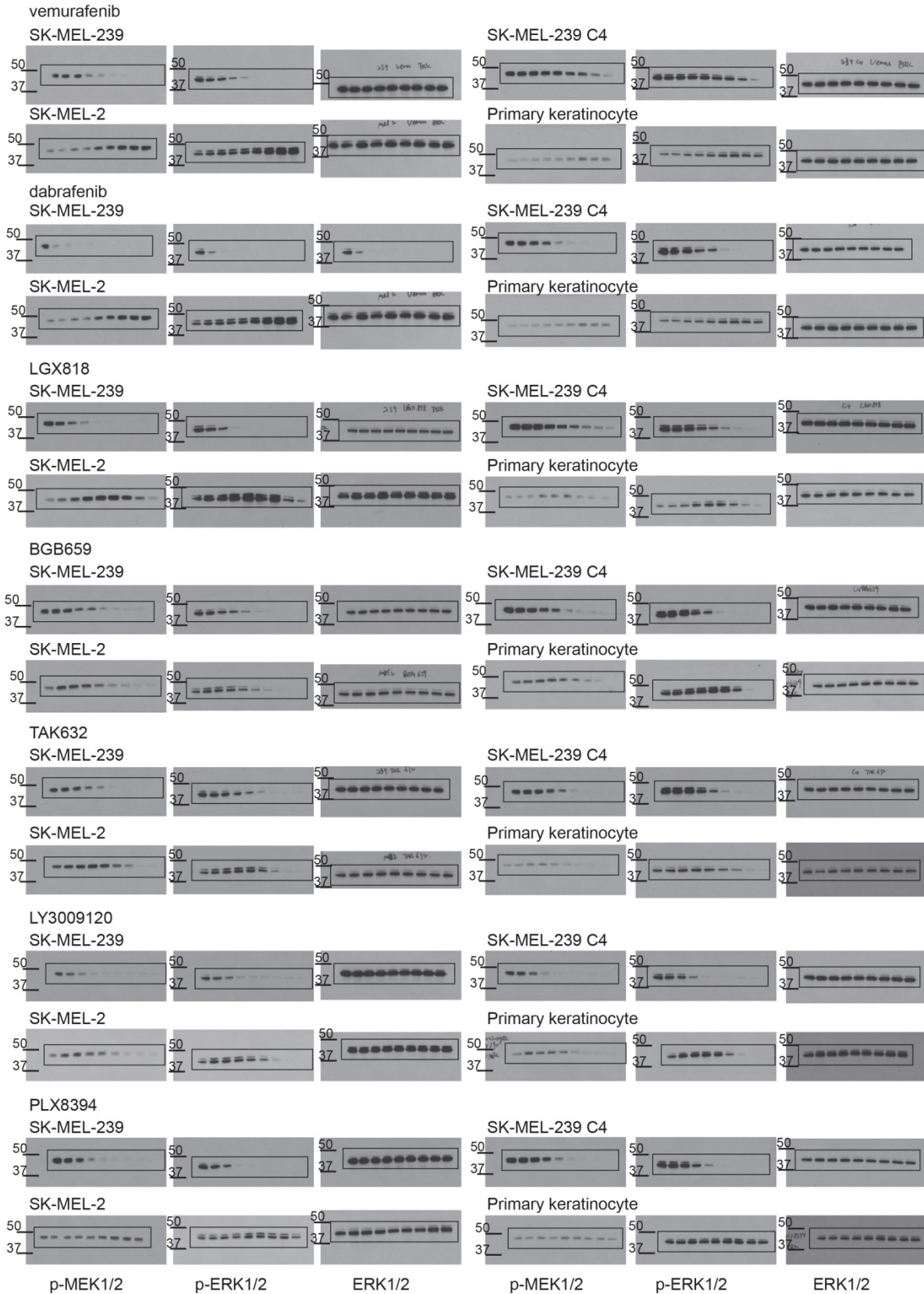


## Supplementary Figure 7

Mutation context	Drug actions	Effect of PLX8394 on ERK signaling	Potential Clinical Effects
<b>Class1 BRAF mutant driven tumors</b> (V600E, V600K, V600D...)		<b>Inhibition</b>	<b>sensitive</b>
<b>Class2 BRAF mutant driven tumors</b> (K601E, L597Q, G469A...)		<b>Inhibition</b>	<b>sensitive</b>
<b>BRAF fusion driven tumors</b>			
<b>BRAF homodimer-dependent acquired resistance of BRAF V600E tumors to current RAFi</b> (Aberrant splicing, intragenic deletion and amplification of BRAF V600E)			
<b>CRAF fusion driven tumors</b>		<b>Activation</b>	<b>insensitive</b>
<b>WT RAF driven tumors</b> (RAS mutants, NF1 loss/mutants or RTKs)		<b>Minimal Effect</b>	<b>insensitive</b>
<b>Normal cells</b>		<b>Minimal Effect</b>	<b>wide therapeutic index</b>



# Supplementary Figure 8





# Supplementary Figure 9

Figure 2

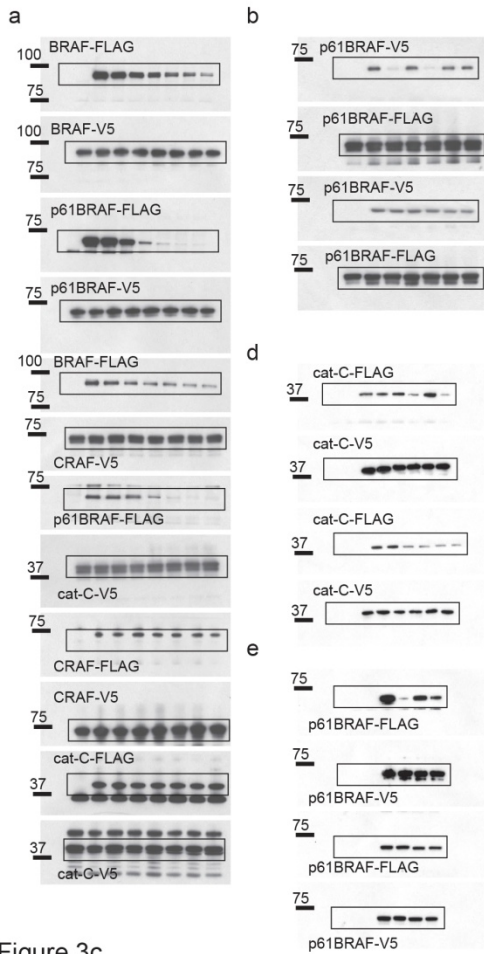


Figure 3c

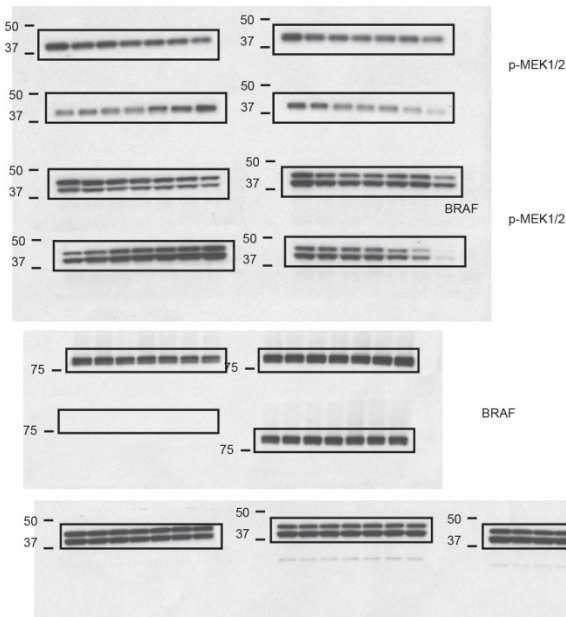


Figure 3a

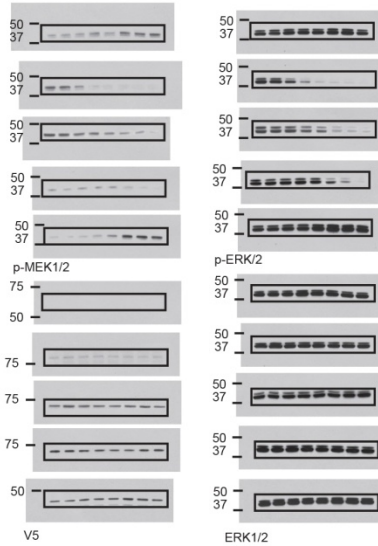
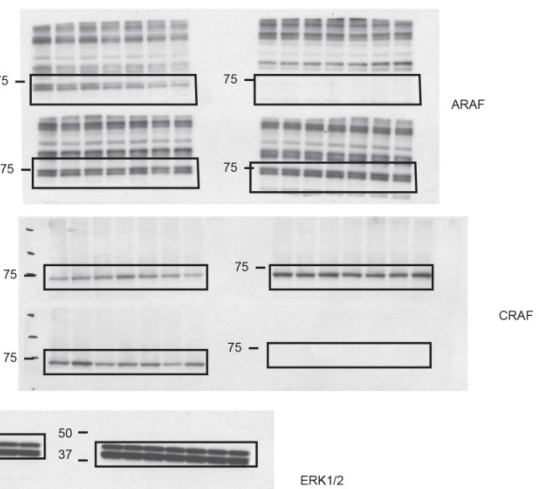
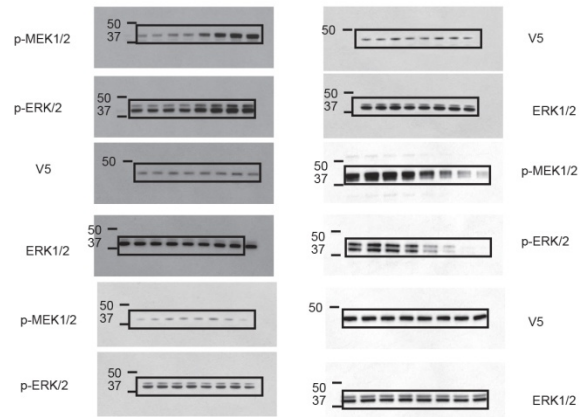
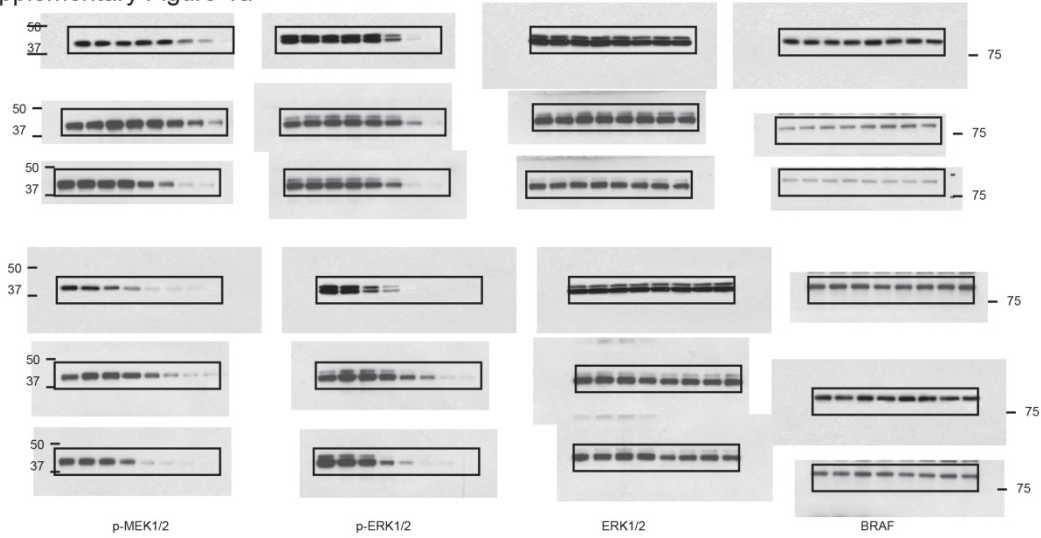


Figure 3b

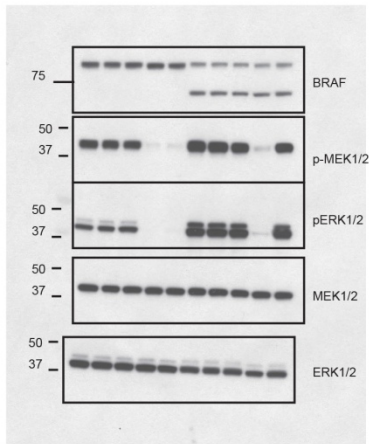


# Supplementary Figure 10

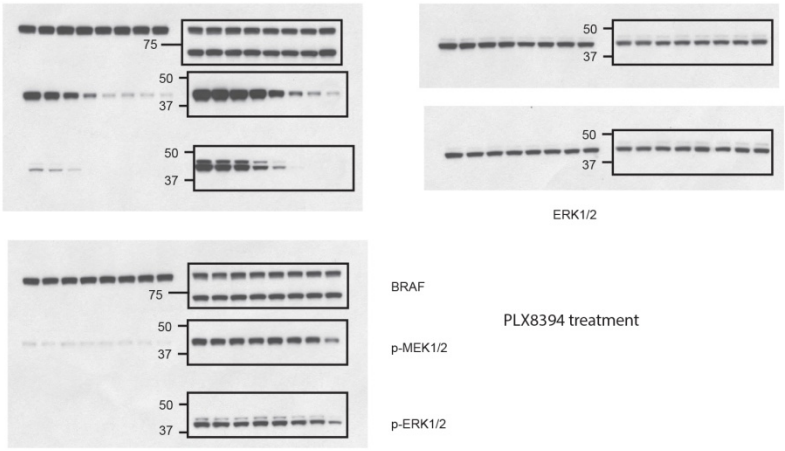
Supplementary Figure 1a



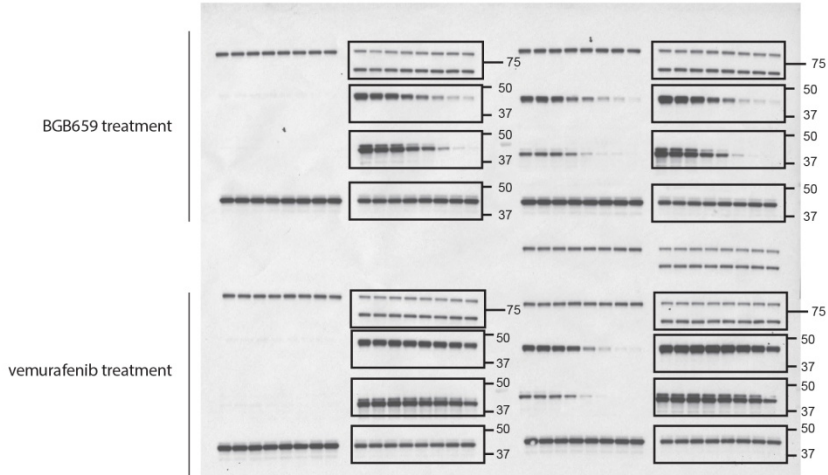
Supplementary Figure 2a



Supplementary Figure 2b

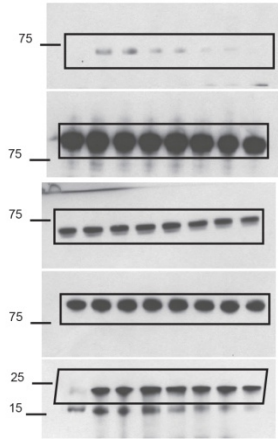


Supplementary Figure 2b

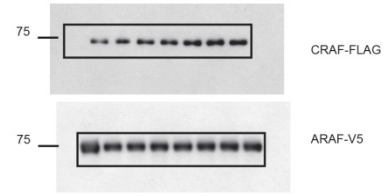
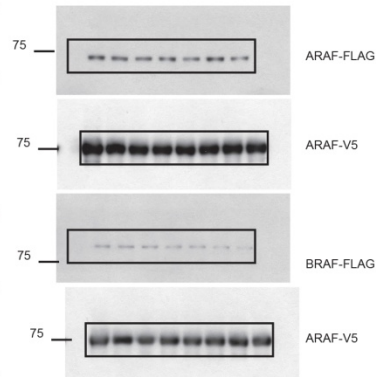


# Supplementary Figure 11

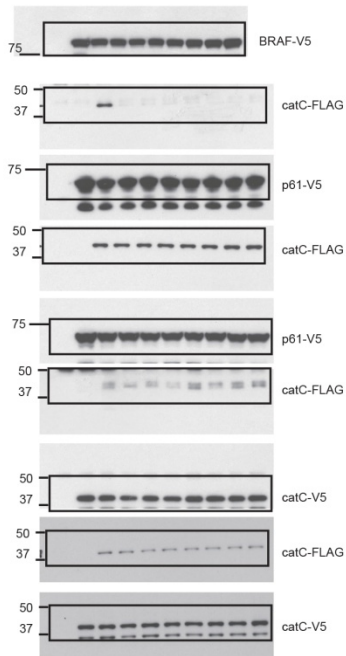
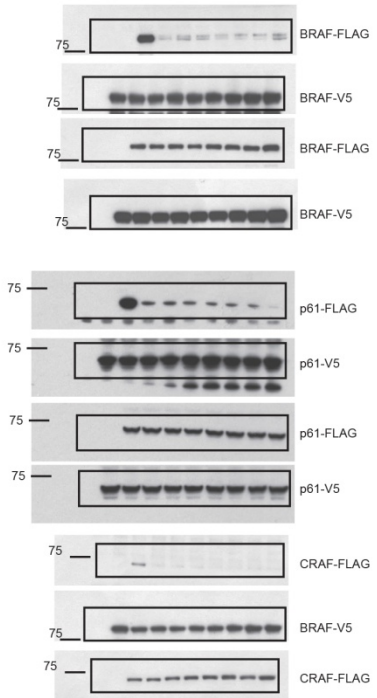
Supplementary Figure 2c



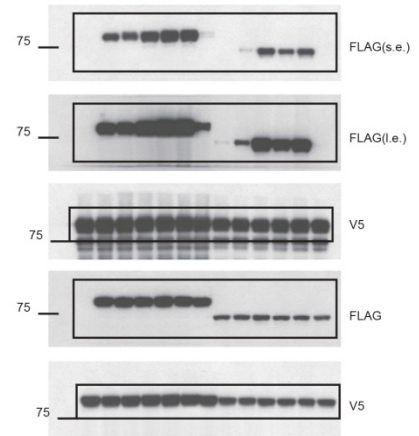
Supplementary Figure 2d



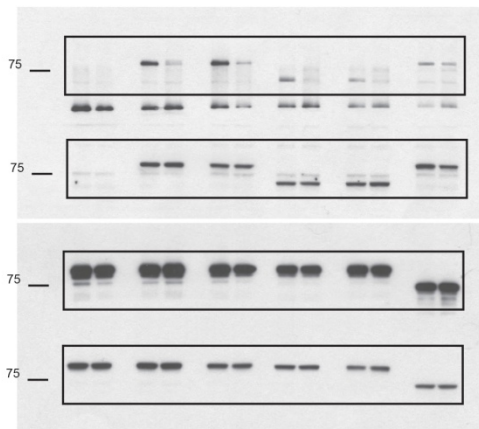
Supplementary Figure 2e



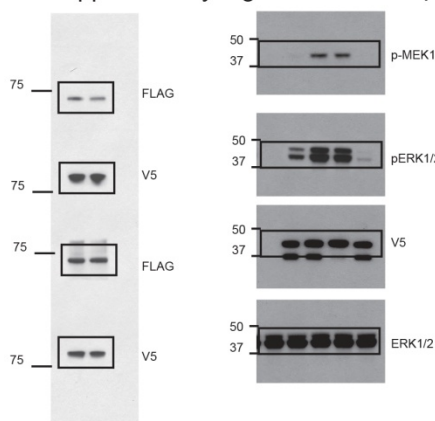
Supplementary Figure 2f



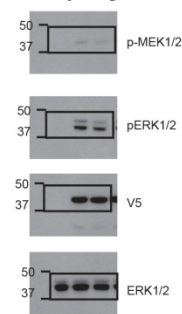
Supplementary Figure 3a



Supplementary Figure 3b



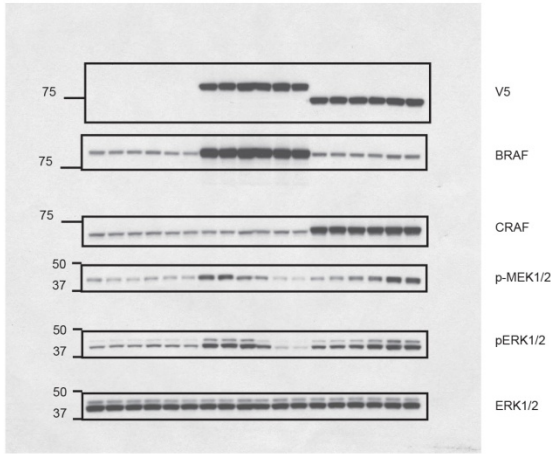
Supplementary Figure 3c



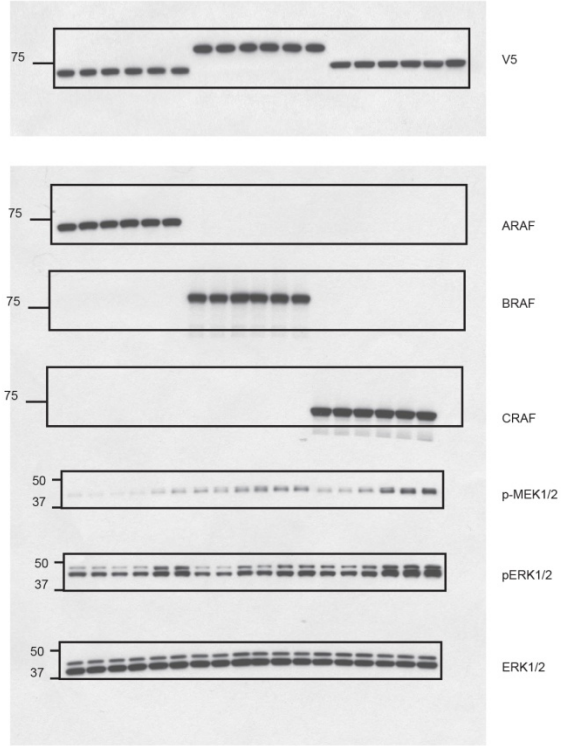


# Supplementary Figure 12

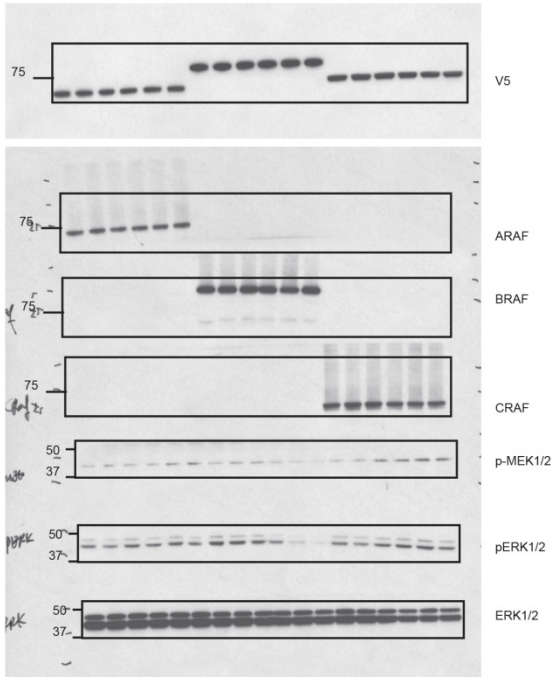
Supplementary Figure 4a



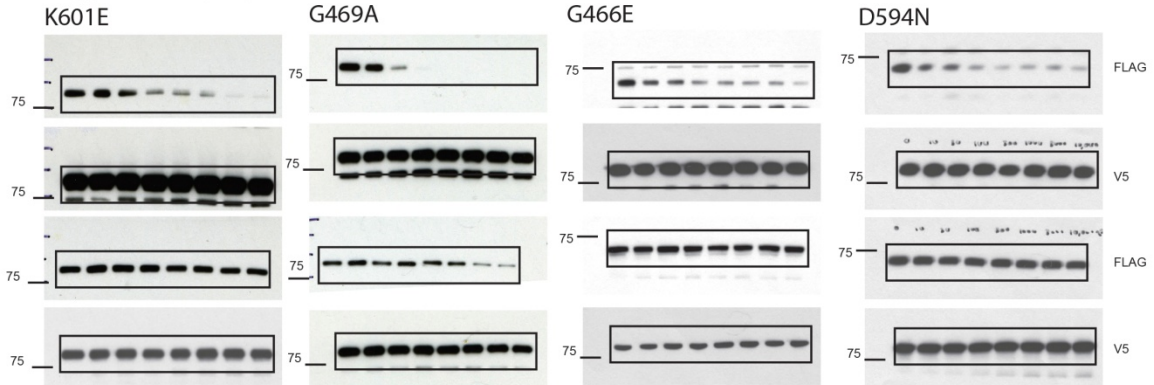
Supplementary Figure 4b



Supplementary Figure 4c



Supplementary Figure 5a

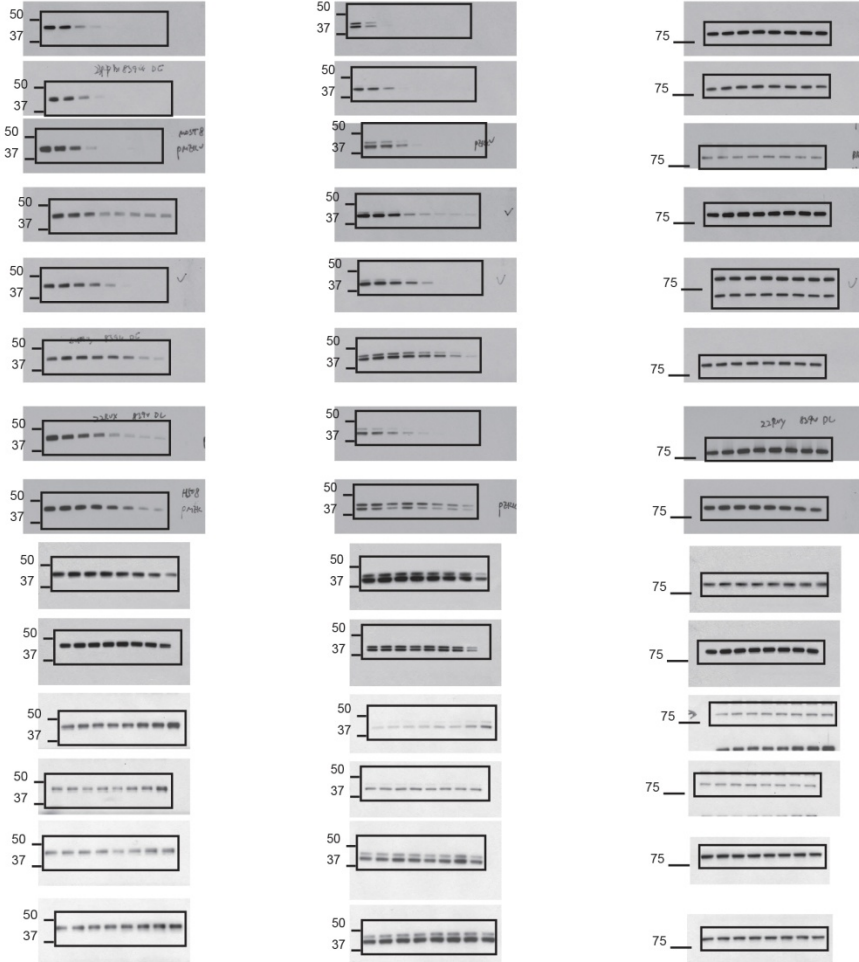




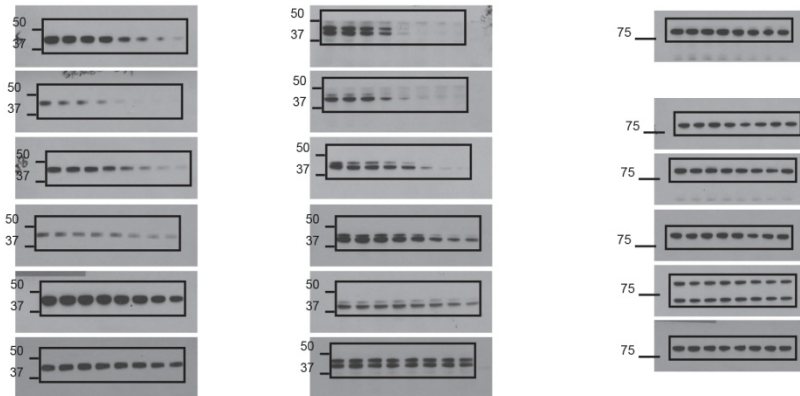
# Supplementary Figure 13

## Supplementary Figure 5b

PLX8394



vemurafenib



p-MEK1/2

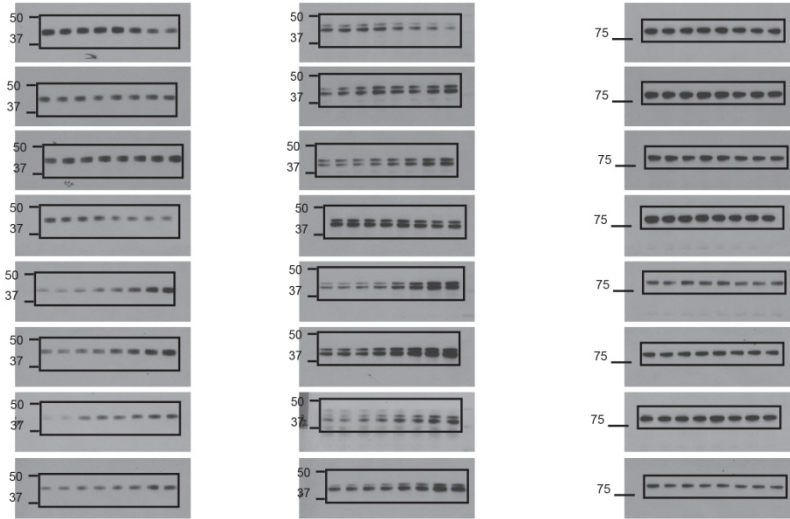
p-ERK1/2

BRAF

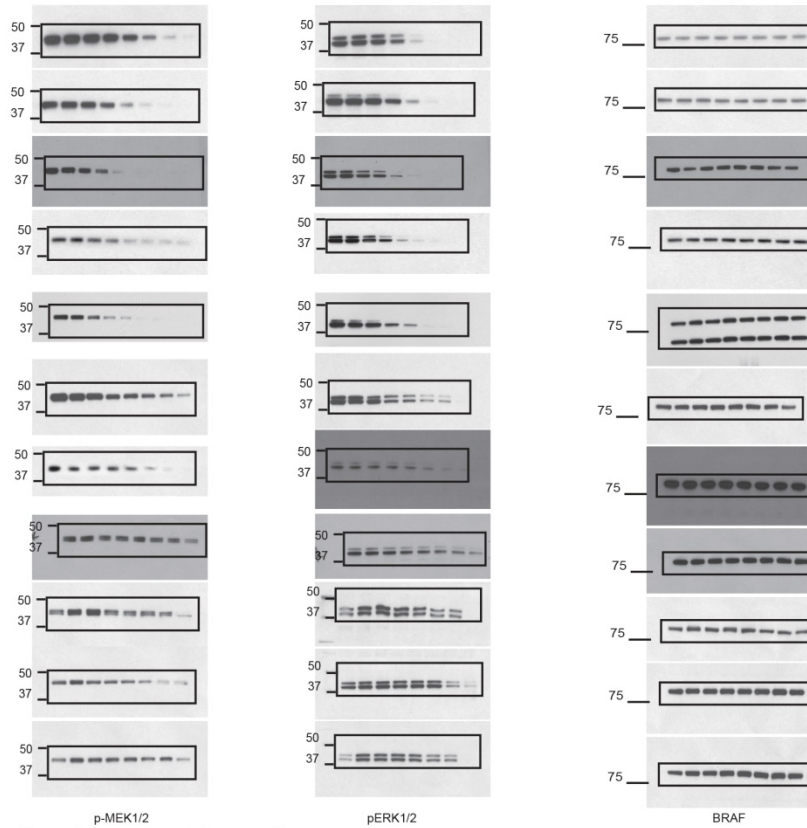
# Supplementary Figure 14

## Supplementary Figure 5b

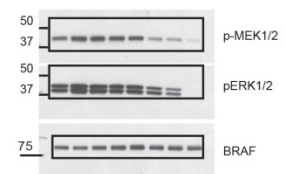
vemurafenib



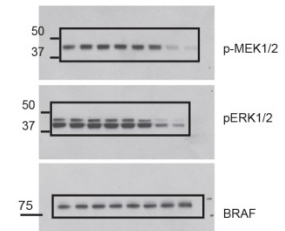
BGB659



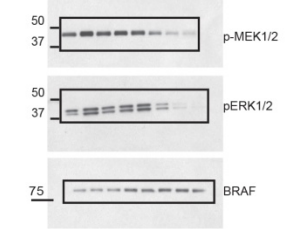
SK-MEL-285



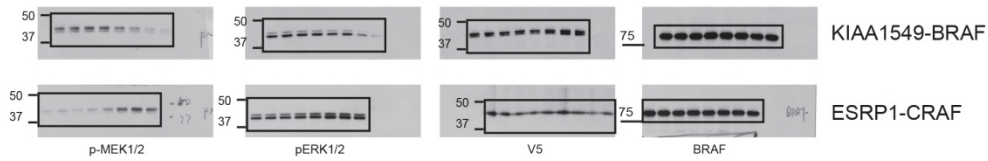
BEAS-2B



primary melanocyte

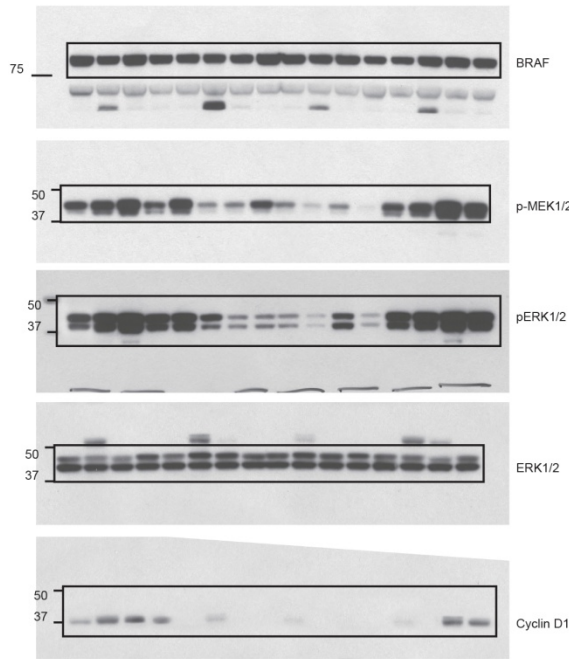


## Supplementary Figure 5c

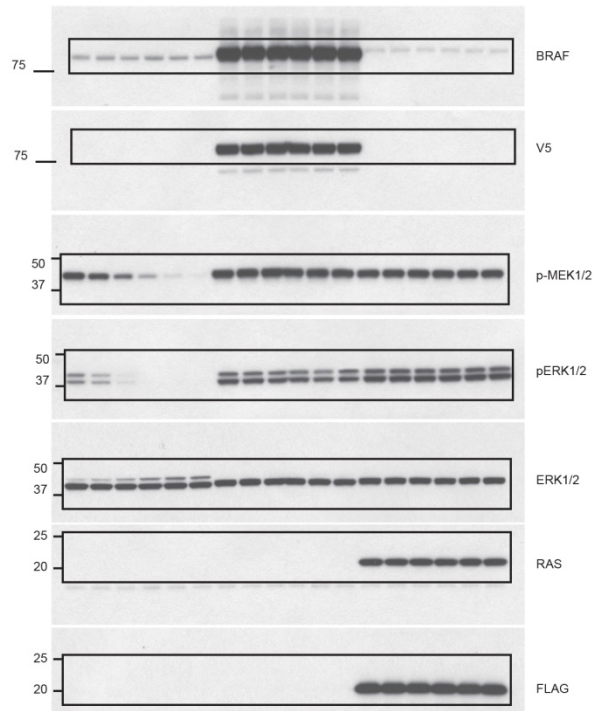


# Supplementary Figure 15

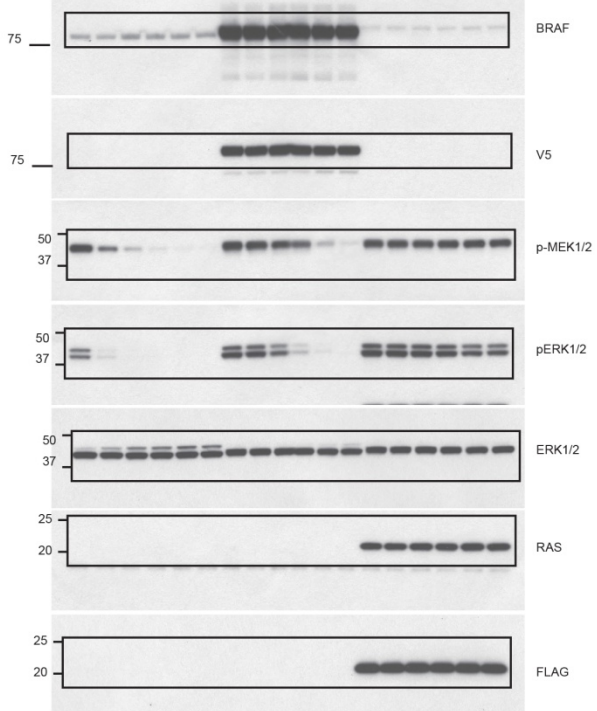
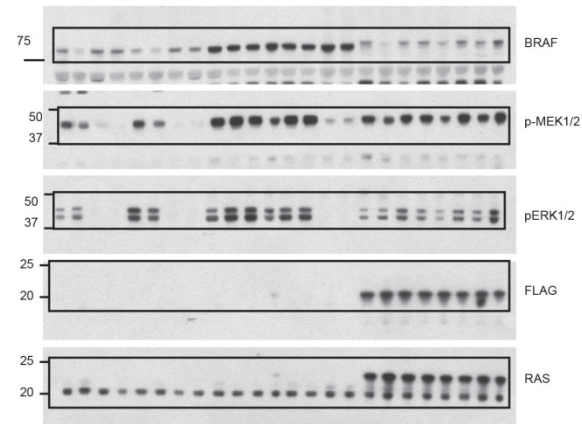
## Supplementary Figure 6a



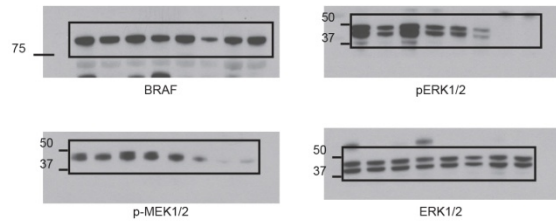
## Supplementary Figure 6b



## Supplementary Figure 6c



## Supplementary Figure 6e



## Supplementary Figure 6f



**Supplementary Table 1. Functional concentrations (nM) of RAF inhibitors (calculated based on the curves in Figure 1)**

	Compound	IC75 of p-ERK in SK-MEL-239	IC75 of p-ERK in SK-MEL-239 C4	EC200 of p-ERK in SK-MEL-2	IC50 of p-ERK in SK-MEL-2	EC200 of p-ERK in Keratinocyte	IC50 of p-ERK in Keratinocyte
Group1	<b>vemurafenib</b>	214	13,490	68	>30,000	790	>30,000
	<b>dabrafenib</b>	25	731	14	25,119	33	20,952
	<b>encorafenib</b>	37	385	11	11,220	60	9,772
Group2	<b>BGB659</b>	309	389	N/A	3,801	N/A	4,074
	<b>TAK632</b>	427	542	N/A	3,311	N/A	6,761
	<b>LY3009120</b>	92	67	N/A	1,218	N/A	2,138
Group3	<b>PLX8394</b>	39	158	N/A	>30,000	N/A	>30,000



**Supplementary Table 2: BRAF-BRAF and CRAF-CRAF dimer interface contacts**

BRAF-BRAF (PDB: 4E26)			CRAF-CRAF (PDB: 3OMV)		
Protomer 2 (Chain B)	Protomer 1 (Chain A)	Number of Contacts <sup>a</sup>	Protomer 2 (Chain B)	Protomer 1 (Chain A)	Number of Contacts <sup>a</sup>
448(ASP)	506(ARG)	4	340(TYR)	398(ARG)	3
			340(TYR)	399(LYS)	10
			341(TYR)	399(LYS)	4
449(ASP)	570(LYS)	1	341(TYR)	462(LYS)	1
450(TRP)	506(ARG)	5	342(TRP)	398(ARG)	5
450(TRP)	507(LYS)	9	342(TRP)	399(LYS)	10
450(TRP)	508(THR)	4	342(TRP)	400(THR)	5
450(TRP)	509(ARG)	20	342(TRP)	401(ARG)	23
450(TRP)	566(TYR)	1	342(TRP)	458(TYR)	2
450(TRP)	570(LYS)	3	342(TRP)	462(LYS)	2
475(LYS)	715(GLU)	1	367(LYS)	607(GLU)	1
476(TRP)	566(TYR)	4	368(TRP)	458(TYR)	2
477(HIS)	510(HIS)	7	369(HIS)	402(HIS)	4
477(HIS)	562(GLN)	14	369(HIS)	454(GLN)	10
477(HIS)	565(ASP)	13	369(HIS)	457(ASP)	8
477(HIS)	566(TYR)	8	369(HIS)	458(TYR)	17
477(HIS)	569(ALA)	2	369(HIS)	461(ALA)	3
478(GLY)	562(GLN)	8	370(GLY)	454(GLN)	8
505(LEU)	509(ARG)	2	397(LEU)	401(ARG)	5
506(ARG)	450(TRP)	5	398(ARG)	342(TRP)	4
506(ARG)	509(ARG)	4	398(ARG)	401(ARG)	8
507(LYS)	448(ASP)	9	399(LYS)	340(TYR)	13
507(LYS)	450(TRP)	7	399(LYS)	342(TRP)	8
508(THR)	450(TRP)	6	400(THR)	342(TRP)	3
508(THR)	509(ARG)	6	400(THR)	401(ARG)	9
509(ARG)	450(TRP)	18	401(ARG)	342(TRP)	23
509(ARG)	505(LEU)	2	401(ARG)	397(LEU)	4
509(ARG)	506(ARG)	4	401(ARG)	398(ARG)	7
509(ARG)	508(THR)	7	401(ARG)	400(THR)	8
509(ARG)	509(ARG)	14	401(ARG)	401(ARG)	24
509(ARG)	515(LEU)	5	401(ARG)	407(LEU)	5
509(ARG)	516(PHE)	15	401(ARG)	408(PHE)	21
509(ARG)	517(MET)	7	401(ARG)	409(MET)	5
510(HIS)	477(HIS)	6	402(HIS)	369(HIS)	2
510(HIS)	515(LEU)	4	402(HIS)	407(LEU)	6
510(HIS)	517(MET)	3	402(HIS)	409(MET)	4
511(VAL)	515(LEU)	1	403(VAL)	407(LEU)	1
511(VAL)	530(GLN)	2	403(VAL)	422(GLN)	4
515(LEU)	509(ARG)	5	407(LEU)	401(ARG)	7

515(LEU)	510(HIS)	3	407(LEU)	402(HIS)	4
515(LEU)	511(VAL)	1	407(LEU)	403(VAL)	2
515(LEU)	515(LEU)	1	407(LEU)	407(LEU)	2
516(PHE)	509(ARG)	17	408(PHE)	401(ARG)	16
517(MET)	509(ARG)	10	409(MET)	401(ARG)	7
517(MET)	510(HIS)	1	409(MET)	402(HIS)	6
530(GLN)	511(VAL)	1	422(GLN)	403(VAL)	4
562(GLN)	477(HIS)	7	454(GLN)	369(HIS)	7
562(GLN)	478(GLY)	8	454(GLN)	370(GLY)	4
565(ASP)	477(HIS)	12	457(ASP)	369(HIS)	10
566(TYR)	450(TRP)	1	458(TYR)	342(TRP)	2
566(TYR)	476(TRP)	3	458(TYR)	368(TRP)	4
566(TYR)	477(HIS)	7	458(TYR)	369(HIS)	13
569(ALA)	477(HIS)	3	461(ALA)	369(HIS)	3
570(LYS)	450(TRP)	2	462(LYS)	340(TYR)	1
570(LYS)	476(TRP)	1	462(LYS)	342(TRP)	4
588(LEU)	586(GLU)	7	480(LEU)	478(GLU)	6
588(LEU)	588(LEU)	1	480(LEU)	480(LEU)	1

<sup>a</sup>Total number of contacts between two residues across the dimer interface. Two atoms are considered in contact if the distance between them is within 4.5Å.

**Supplementary Table 3. Mutation profiles of patient tumors and PDX samples.**

BRAF G466V CRC TUMOR		BRAF K601E CRC TUMOR	
Mutations in patient sample	Mutation in PDX sample	Mutations in patient sample	Mutation in PDX sample
BRAF G466V	BRAF G466V	BRAF K601E	BRAF K601E
TP53 A161S	TP53 A161S	TP53 R248Q	TP53 R248Q
APC L1488fs	APC L1488fs	APC T1566fs	APC T1566fs
APC S794fs	APC Y796fs	MLL2 T2524M	MLL2 T2524M
BARD1 Q237X	BARD1 Q237X	MAP2K1 V211D	MAP2K1 V211D
FLCN S108I	FLCN S108I	ATRX M734I	ATRX M734I
LATS1 R287Q	LATS1 R287Q	AR Q671R	FBXW7 DEL
MITF Q2P	MITF Q2P	FBXW7 DEL	ZRSR2 S447_R448dup
MYCL1 Y43C	MYCL1 Y43C	MYC AMP	PAK1 N228T
MAP2K4 Intragenic deletion (17p)	TGFBR1 A24_A26 del	CDKN1B AMP	ZFHX3 Q3204dup
PARK2 Intragenic deletion	ARID1B Q633L	NOTCH3 G2038D	BCOR R170K
PIK3R1 Intragenic deletion	TGFBR2 deletion	PTPRS P1418L	
	MAP2K4 Intragenic deletion (17p)	ZRSR2 S447_R448dup	
		PAK1 N228T	
		ZFHX3 Q3204dup	
		BCOR R170K	

**Supplementary Table 4. Properties of RAF inhibitors.**

<b>compounds</b>	<b>Type of ATP competitive drug</b>	<b>Selectivity In tumors</b>	<b>Sensitive to Negative Cooperative Effect</b>	<b>activity on RAF dimers</b>	<b>Paradoxical activation</b>
<b>vemurafenib</b>	Group 1	BRAF V600 mutant monomer	Yes	No	Strong
<b>dabrafenib</b>		BRAF V600 mutant monomer	Yes	No	Strong
<b>encorafenib</b>		BRAF V600 mutant monomer	Yes	No	Strong
<b>TAK632</b>	Group 2	Mutant RAF (A/B/C) monomer and dimer	No	Yes	Low
<b>LY3009120</b>		Mutant RAF (A/B/C) monomer and dimer	No	Yes	Low
<b>BGB659</b>		Mutant RAF (A/B/C) monomer and dimer	No	Yes	Low
<b>PLX8394</b>	Group 3	Ras-independent BRAF mutant monomer and dimer	Yes	Selectively disrupts BRAF-containing dimers	Very low



## Supplementary Figure Legends

**Supplementary Figure 1. ERK signaling responses to RAF inhibitors in wild type or mutant BRAF cell lines.** (a) A375, SK-MEL-30 (NRAS Q61K) and encorafenib pretreated SK-MEL-30 cells (treated with 1  $\mu$ M encorafenib for 1 hr, followed by drug washout with drug free medium<sup>1</sup>) were treated with indicated drugs at increasing doses for 1hr. The ERK signaling response was tested by Western blot. These experiments were repeated 3 times. For gel source data, see Supplementary Figure 10. (b) SK-MEL-239, SK-MEL-239 C4, SK-MEL-2 and primary keratinocyte cells were treated with the indicated drugs at increasing doses for 3 days. The growth response was determined by ATP-Glo assay. Dose dependent inhibition curves were generated using Prism6 (mean $\pm$ s.d. are represented by the dots and error bars, n=8).

**Supplementary Figure 2. PLX8394 selectively disrupts BRAF containing dimers.** (a) SK-MEL-239 parental cells and SK-MEL-239 C4 cells were treated with DMSO or encorafenib for 1hr, or followed by drug washout and cultured in fresh medium for another hour. The samples were collected and assayed by Western blot with indicated antibodies. (b) SK-MEL-239 C4 cells were treated with DMSO or encorafenib for 1hr, and then the cells were washed with drug free medium and cultured in fresh medium with vemurafenib, BGB659 or PLX8394 at increasing doses for an additional hour. The ERK signaling response was tested by Western blot. (c) NRAS mutant expression was induced by doxycycline treatment in 293H (NRAS Q61K) cells for 1 day, the cells were then treated with indicated doses of PLX8394 for 1hr. The BRAF/CRAF complex was pulled down by co-immunoprecipitation with anti-BRAF antibody. The interaction between BRAF and CRAF were assayed by Western blot with indicated antibodies. (d) 293H (NRAS Q61K) cells were co-transfected with pcDNA3-FLAG vector plus pcDNA3-ARAF-V5 or pcDNA3-ARAF-FLAG plus pcDNA3-ARAF-V5, pcDNA3-FLAG vector plus pcDNA3-ARAF-V5 or pcDNA3-BRAF-FLAG plus pcDNA3-ARAF-V5 and pcDNA3-FLAG vector plus pcDNA3-ARAF-V5 or pcDNA3-CRAF-FLAG plus pcDNA3-ARAF-V5 plasmids. After 24 hrs, the cells were treated with DMSO, or PLX8394 at indicated concentrations for 1hr. Then the cell lysates were extracted and immunoprecipitated with anti-V5 agarose. The input and IP samples were analyzed by Western blot with the indicated antibodies. (e) 293H cells ectopically expressing the indicated RAF proteins were treated with 1 $\mu$ M of PLX8394 for increasing periods of time before lysis. Then the cell lysates were assayed by immunoprecipitation with anti-V5 agarose. The input and IP samples were analyzed by Western blot with anti-FLAG or anti-V5 antibodies. (f) 293H (NRAS Q61K) cells ectopically expressing the indicated RAF proteins were lysed after 1hr treatment with DMSO or different RAF inhibitors (1 $\mu$ M). Then the cell lysates were assayed by immunoprecipitation with anti-V5 agarose. The input and IP samples were analyzed by Western blot with anti-FLAG or anti-V5 antibodies. All the experiments results shown here were repeated for 4 times, independently. For gel source data, see Supplementary Figure 10&11.

**Supplementary Figure 3. PLX8394 disrupts BRAF containing dimers by binding to only one protomer in the dimers.** (a) 293H cells ectopically expressing the indicated RAF proteins were lysed after 1hr treatment with DMSO or PLX8394. Then the cell lysates were immunoprecipitated with anti-V5 agarose. The input and IP samples were analyzed by Western blot with anti-FLAG or anti-V5 antibodies. (b) and (c) the truncated RAF proteins as indicated were transiently expressed in 293H cells for 1 day, then the cells were cultured in serum free medium for 6hrs and collected. Expression and/or phosphorylation of the indicated proteins were assayed by Western blot. All the experiments results shown here were repeated for 3 times, independently. For gel source data, see Supplementary Figure 11.

**Supplementary Figure 4. PLX8394 selectively inhibits BRAF driven ERK signaling activation** (a) HeLa cells stably expressing vector, BRAF-V5 or CRAF-V5 were treated with PLX8394 at the indicated doses for 1hr. The ERK signaling response was tested by Western blot. (b) & (c) The conditional RAF-less A-Raf<sup>lox/lox</sup>; B-Raf<sup>lox/lox</sup>; c-Raf<sup>lox/lox</sup>; RERT<sup>ert/ert</sup> MEF cells were generated by the Barbacid lab as described previously. We introduced doxycycline controlled expression of ARAF, BRAF or CRAF in these cells when the endogenous ARAF/BRAF/CRAF genes were removed by Cre expression. Then the cells were treated with vemurafenib (b) or PLX8394 (c) at the indicated doses for 1hr. The ERK signaling response was tested by Western blot. All the experiments results shown here were repeated for 4 times, independently. For gel source data, see Supplementary Figure 12.

**Supplementary Figure 5. PLX8394 preferentially inhibits activating BRAF mutants or fusions driven ERK signaling in cells.** (a) 293H cells ectopically expressing the indicated RAF proteins were lysed after 1hr treatment with increasing doses of PLX8394. Then the cell lysates were assayed by immunoprecipitation with anti-V5 agarose. The input and IP samples were analyzed by Western blot with anti-FLAG or anti-V5 antibodies. (b) Effect on ERK signaling in a panel of cell lines exposed to PLX8394, vemurafenib or BGB659 for 1 hour at indicated doses. Cell lines in which the ERK signaling is driven by different classes of BRAF mutants are labeled in different colors. Black: Class 1 BRAF mutants (V600 mutants), blue: Class 2 BRAF mutants, and red: Class 3 BRAF mutants. Names of RAS mutant cell lines are labeled with purple color. Names of WT RAS/RAF lines are in green color. (c) The indicated BRAF or CRAF fusion proteins were ectopically expressed in 293H cells. The cells were treated with PLX8394 at the indicated doses for 1 hr. The ERK signaling response was tested by Western blot. All the experiments results shown here were repeated for 3 times, independently. For gel source data, see Supplementary Figure 12, 13&14.

**Supplementary Figure 6. In vivo activity of PLX8394 in tumors driven by BRAF mutants.** (a) Mice carrying A375 Xenograft tumors were treated with 50mg/kg PLX8394 (i.p.) once the tumors grew to about 100mm<sup>3</sup>. The tumor samples at each time point after drug injection were collected (n=2). The drug concentration in the tumor tissues and ERK pathway activation were examined. Each column represents the test result of a tumor sample. The different colors indicate the two experimental groups. (b) A375 cells stably expressing GFP, BRAF V600E and NRAS Q61K were treated with vemurafenib or PLX8394 at the indicated doses for 1hr. ERK signaling was assayed by Western blot. The experiments results shown

here were repeated for 3 times, independently. (c) The A375 control, A375 with BRAF V600E overexpression or A375 with NRAS Q61K expression Xenograft tumors were treated with vehicle, vemurafenib or PLX8394. 6hrs after one dose treatment, the tumors (n=2 in each group) were collected and lysed. ERK signaling was assayed by Western blot. (d) The BRAF K601E CRC PDX tumors established from tumor biopsy specimens were treated with the indicated drugs. Response was monitored by measurement of the tumor sizes. Vemurafenib was given at 75 mg/kg BID and PLX8394 at 50 mg/kg BID. The curves for tumor growth were generated using Prism6. Tumor sizes are shown as mean±s.d. in the graphs (n=5, p-value was calculated by two-tailed unpaired t-test). (e) & (f) The BRAF K601E and the BRAF G466V PDX tumors were treated with one dose of the indicated drugs. Six hours after the treatment the tumor samples were collected and lysed (n=2 in each group). ERK signaling was assayed by Western blot. For gel source data, see Supplementary Figure 15.

### **Supplementary Figure 7. Effects of PLX8394 in tumors as a function of the mechanisms of activation of RAS-RAF signaling**

The effects of PLX8394 in cells are determined by their genotypes, levels of expression of RAF family members, and mechanism of activation of ERK signaling. In tumor cells with activating BRAF mutants or fusions, the ERK pathway is hyperactivated, and therefore, RAS activity and RAS-dependent WT RAF dimers are feedback inhibited. In these tumors, ERK signaling is dominated by mutant BRAF monomers (Class1 mutants) or homodimers (Class 2 BRAF mutants, RAF fusions, N-terminal deletions, and splicing variants, and significantly overexpressed Class 1(BRAF V600) mutants. PLX8394 potently inhibits ERK activation and tumor growth by directly binding to and inhibiting mutant BRAF monomers or by binding to and disrupting BRAF homodimers, BRAF/CRAF heterodimers and BRAF dimeric fusion proteins. By contrast, CRAF homodimers and ARAF containing dimers are not disrupted by the drug. Hence, normal cells and tumors in which RTKs, RAS mutation, NF1 loss, or CRAF fusions drive ERK signaling ought to be insensitive to the drug. In normal cells in which ARAF, BRAF and CRAF are all expressed, PLX8394 disrupts BRAF/BRAF and BRAF/CRAF dimers; but transactivates CRAF homodimers and ARAF dimers. Thus, the opposing effects of PLX8394 on different dimers tend to prevent significant inhibition or paradoxical activation of the ERK signaling. This suggests that this drug will have a wide therapeutic index.

### **Supplementary Figure 8. Source data for Figure 1**

### **Supplementary Figure 9. Source data for Figure 2 and Figure 3**

**Supplementary Figure 10. Source data for Supplementary Figure 1 and 2**

**Supplementary Figure 11. Source data for Supplementary Figure 2 and 3**

**Supplementary Figure 12. Source data for Supplementary Figure 4 and 5**

**Supplementary Figure 13. Source data for Supplementary Figure 5**

**Supplementary Figure 14. Source data for Supplementary Figure 5**

**Supplementary Figure 15. Source data for Supplementary Figure 6**

## **References**

1. Yao, Z., *et al.* BRAF Mutants Evade ERK-Dependent Feedback by Different Mechanisms that Determine Their Sensitivity to Pharmacologic Inhibition. *Cancer Cell* **28**, 370-383 (2015).