Supplemental Methods

Reagents

Phospho-AKT at T308 (pAKT T308), phospho-AKT at S473 (pAKT S473), AKT, ERK, phospho-S6P at S235/236 (pS6P), S6P, cyclin D1, phospho-MEK1/2 at S217/S221 (pMEK), MEK1/2, MEK1, MEK2, and cleaved PARP (cPARP) antibodies were purchased from Cell Signaling. Primary antibodies to GAPDH, RAF-1 (CRAF), BRAF, and ARAF were purchased from Santa Cruz Biotechnology. Diphospho-ERK1/2 at T202 and Y204 (ppERK) antibody was purchased from Sigma and $p27^{kip1}$ antibody was purchased from Epitomics. Anti-Mouse IRDye® 800CW antibody was purchased from LI-COR Biosciences. Anti-rabbit Alexa Flour® 680 antibody was purchased from Invitrogen. RPMI 1640 media was obtained from Invitrogen. Fetal Bovine Serum (FBS) was purchased from Hyclone.

Sanger genomic DNA sequencing

Genomic DNA (gDNA) isolation was prepared using the Promega Maxwell 16 Cell DNA Purification Kit (Promega, Madison WI). All gDNA PCR primers were ordered from IDT (Integrated DNA Technologies Inc, Coralville, IA). PCR primers were tailed with M13 universal sequencing primer sequences. PCR reactions were carried out using Roche FastPCR DNA polymerase (Roche, Indianapolis, IN). DNA was amplified for 35 cycles at 95ºC for 20 seconds, 60ºC for 30 seconds and 72ºC for 45 seconds followed by 7 minutes extension at 72ºC. All gDNA primers have been tested for quality control (QC) on Promega Human Genomic DNA (Promega, Madison WI), before using on cell line samples. PCR products were purified using Agencourt AmPure (Agencourt Bioscience Corporation, Beverly, MA). Direct sequencing of purified PCR products was performed

with AB v3.1 BigDye-terminator cycle sequencing kit (Applied Biosystems, Foster City, CA) and sequencing reactions were purified using Agencourt CleanSeq (Agencourt Bioscience Corporation, Beverly, MA). The sequencing reactions were analyzed using a Genetic Analyzer 3730XL (Applied Biosystems, Foster City, CA). All sequence results data were assembled and analyzed using Codon Code Aligner (CodonCode Corporation, Dedham, MA). The primer sequences are:

BRAF.NM_004333.4	
Exon01 forward	TGTAAAACGACGGCCAGTCCGGCTCTCGGTTATAAGATG
Exon01 reverse	CAGGAAACAGCTATGACCAAATAAACACCAGCCAGCCG
Exon02 forward	TGTAAAACGACGGCCAGTGGAAGGCTGCTCACCAAACCA
Exon02 reverse	CAGGAAACAGCTATGACCGGCAAAGCTAATTCTCTCTTCCCA
Exon03 forward	TGTAAAACGACGGCCAGTTGTCAGGACAAAGTCCGGATTGA
Exon03 reverse	CAGGAAACAGCTATGACCGACATGACTGTGGTTCAAGTTTGGC
Exon04 forward	TGTAAAACGACGGCCAGTTGATGGATATACTGCGTTGGTGGG
Exon04 reverse	CAGGAAACAGCTATGACCTGGCCTACAGTATTTCTTCAGGCTAAC
Exon04b forward	TGTAAAACGACGGCCAGTGTTGTATCTGACCTAGTAAC
Exon04b reverse	CAGGAAACAGCTATGACCCCTACAGTATTTCTTCAGGC
Exon05 forward	TGTAAAACGACGGCCAGTTAATAACCAAGAAAGGCTTG
Exon05 reverse	CAGGAAACAGCTATGACCATTCATTCATTAAAATCTAA
Exon06 forward	TGTAAAACGACGGCCAGTGCTTGAAATCAGTTGCCAGCCT
Exon06 reverse	CAGGAAACAGCTATGACCTGGGAGAGAAATACTGTCCATTCCAC
Exon07 forward	TGTAAAACGACGGCCAGTAAATTTCTCTTTGCTGTATGCCAGTTT

Exon07 reverse	CAGGAAACAGCTATGACCCCCAAGAGCAGAAGTCAAACCA
Exon08 forward	TGTAAAACGACGGCCAGTCCTGGAGGGCCTACTTGAGCA
Exon08 reverse	CAGGAAACAGCTATGACCAATGTCATCAGAGAGAAACCAGAAGC
Exon09 forward	TGTAAAACGACGGCCAGTTGGCCTGAAATGGACATCAACA
Exon09 reverse	CAGGAAACAGCTATGACCTGTGTCTGTTACTTGAAAGAGGCAAG
Exon10 forward	TGTAAAACGACGGCCAGTTTTGGCCCATCCTTTCCAA
Exon10 reverse	CAGGAAACAGCTATGACCTGAAATATCTGAATTCTGGACCAGCC
Exon11 forward	TGTAAAACGACGGCCAGTTCTCTTCCTGTATCCCTCTCAGGCA
Exon11 reverse	CAGGAAACAGCTATGACCGGAGTCCCGACTGCTGTGAA
Exon12 forward	TGTAAAACGACGGCCAGTCCATGGAACAAACAAGGTTGGC
Exon12 reverse	CAGGAAACAGCTATGACCGCTACCACTGGGAACCAGGAGC
Exon13 forward	TGTAAAACGACGGCCAGTAAGCAACCTCTAGTTGCCTGTCAGA
Exon13 reverse	CAGGAAACAGCTATGACCTCTGAAGGCTGCACTAGACAGAGACA
Exon14 forward	TGTAAAACGACGGCCAGTGGCTTGACTGGAGTGAAAGGTTTG
Exon14 reverse	CAGGAAACAGCTATGACCCAGGCTGTGGTATCCTGCTCTCC
Exon15 forward	TGTAAAACGACGGCCAGTCATAATGCTTGCTCTGATAG
Exon15 reverse	CAGGAAACAGCTATGACCTAACTCAGCAGCATCTCAGG
Exon16 forward	TGTAAAACGACGGCCAGTCAGGAATGGGAAGTGGGAACTGA
Exon16 reverse	CAGGAAACAGCTATGACCTCCTTCACGCTTACCCAGGAGTT
Exon17 forward	TGTAAAACGACGGCCAGTTTTGTGGGTTTCCCACCATCT
Exon17 reverse	CAGGAAACAGCTATGACCTGCTCAACCCTCATGAAGCCA
Exon18 forward	TGTAAAACGACGGCCAGTGGTGCTTTCTTGTAAAGTGTGATGG

Exon18 reverse	CAGGAAACAGCTATGACCCAAACCCGGAACAGAAAGTAAAGC
Exon18b forward	TGTAAAACGACGGCCAGTGTGTGATGGGACTCTTAAAG
Exon18b reverse	CAGGAAACAGCTATGACCGTTGCTACTCTCCTGAACTC
Exon18c forward	TGTAAAACGACGGCCAGTGTAGATTCTCGCCTCTATTG
Exon18c reverse	CAGGAAACAGCTATGACCGTTGCTACTCTCCTGAACTC

MEK1.NM_002755.3	
Exon 01 forward	tgtaaaacgacggccagtAGCGGGAGGAAGCGAGAGGT
Exon 01 reverse	caggaaacagctatgaccCCCAGACGTACGTGACAAACCA
Exon 02 forward	tgtaaaacgacggccagtCGGGTGGCTGGAGTGAAGTG
Exon 02 reverse	caggaaacagctatgaccCAGTCTTCCTTCTACCCTGGTCCC
Exon 03 forward	tgtaaaacgacggccagtCCGAGGCACAGCTGGAACAG
Exon 03 reverse	caggaaacagctatgaccGGAGGCCTGCCATCTTGTGA
Exon 04 forward	tgtaaaacgacggccagtCATAGGACAAGGTATGGGAGTGGG
Exon 04 reverse	caggaaacagctatgaccTCCAGACAGGGAAGAGTCTAGTGAGAA
Exon 05 forward	tgtaaaacgacggccagtGGAGGAAGGCAAATTTGTGATGA
Exon 05 reverse	caggaaacagctatgaccAGCTGGCCAGTCCTGGCTTT
Exon 06 forward	tgtaaaacgacggccagtCCAAGGGCTGCCTCTGATGG
Exon 06 reverse	caggaaacagctatgaccGCCACACTCTGGAGCTGGACA
Exon 07 forward	tgtaaaacgacggccagtCAGCCAGGGCACAGCTGATT
Exon 07 reverse	caggaaacagctatgaccTAGCCTGGCATCCAGGGACA
Exon 08 forward	tgtaaaacgacggccagtTGTGGCTGTTTAATGTTTATTGTCCA

Exon 08 reverse	caggaaacagctatgaccTGAACCTGTGACAGCGATGTGTT
Exon 09 forward	tgtaaaacgacggccagtTACGTTCCCATGCCGCACTC
Exon 09 reverse	caggaaacagctatgaccTCAGACGGGAGGGTAAAGGGC
Exon 10 forward	tgtaaaacgacggccagtGGATGAATCAAGCCCAGGCA
Exon 10 reverse	caggaaacagctatgaccTGCCACCAAACGAGACAGCA
Exon 11 forward	tgtaaaacgacggccagtCACCACGTCCTCTCGTTTCCTT
Exon 11 reverse	caggaaacagctatgaccAGGATCTCACAAGGCTCCCTCC

MEK2.NM_030662.3	
Exon 1 forward	tgtaaaacgacggccagtCTATGGGCCCCGGCTAGAGG
Exon 1 reverse	caggaaacagctatgaccGCCTCGTGCACTCCTCGCGAA
Exon 2 forward	tgtaaaacgacggccagtCCCAGAAGCTGGGCCCTTTC
Exon 2 reverse	caggaaacagctatgaccCCGCCCTGGATTCTGGTCAT
Exon 3 forward	tgtaaaacgacggccagtTGGCATCGACTGCCTTGAGAA
Exon 3 reverse	caggaaacagctatgaccTGACCACTGTTGGGAACGCC
Exon 4 forward	tgtaaaacgacggccagtAGGTCCTGGCGTCCCACATC
Exon 4 reverse	caggaaacagctatgaccCGTGGGTGGAGTTGGGCTC
Exon 4b forward	tgtaaaacgacggccagtACGCATCCGTGGCTTCCCG
Exon 4b reverse	caggaaacagctatgaccGGCAGATGGTGACCTCAGGC
Exon 5 and 6 forward	tgtaaaacgacggccagtGTTGGGAAAGCCTCGTGGGA
Exon 5 and 6	caggaaacagctatgaccGCGAGCTCCTCACAGCCTGA

reverse	
Exon 5b forward	tgtaaaacgacggccagtCAGCTCTGACTGCTCAGCTC
Exon 5b reverse	caggaaacagctatgaccGCCATGGAGTCGATGAGCTG
Exon 6b forward	tgtaaaacgacggccagtCAGATCATGCACCGAGGTAAG
Exon 6b reverse	caggaaacagctatgaccGACAGCTGCGCAGGAGACATG
Exon 7 forward	tgtaaaacgacggccagtGACCACAGTCGGCACCATCC
Exon 7 reverse	caggaaacagctatgaccCTGCGGTCAACACCAGCTCC
Exon 8 forward	tgtaaaacgacggccagtGGGAAGGAGCTGGGCTTGTG
Exon 8 reverse	caggaaacagctatgaccCGCCTCCAATTTAGGCTGGC
Exon 8b forward	tgtaaaacgacggccagtCAGTGCCTCTGCACACCCGC
Exon 8b reverse	caggaaacagctatgaccCTCTTGCTCTGGTCAGAGAG
Exon 9 forward	tgtaaaacgacggccagtACAGGGCCTGCAATCCTGCT
Exon 9 reverse	caggaaacagctatgaccAGTTGGCATGTGGTGTCGGC
Exon 10 forward	tgtaaaacgacggccagtCCAGAGCTGTTCCCACGCAC
Exon 10 reverse	caggaaacagctatgaccCCCTGGCACCAAAGTGCAGA
Exon 11 forward	tgtaaaacgacggccagtGCTCAGGGATGTCCTCTCCG
Exon 11 reverse	caggaaacagctatgaccGGAAGGGAGGGTCTCCTCTGTTT
Exon 11b forward	tgtaaaacgacggccagtGTCCTACTGTGACTCCAGG
Exon 11b reverse	caggaaacagctatgaccGCCAGCCTGTCCTCAGCTG

Single nucleotide polymorphism (SNP) analysis

Genomic DNA was purified from A375 and all of the A375 resistant clones. DNA was labeled and hybridized for SNP analysis on Affymetrix Genome Wide Human SNP Array

6.0 chips according to the manufacturer's instructions. Data analysis was performed with Array Studio. The A375 resistant clones shared greater than 95% similarly to A375 as determined by identical by state and identical by decent calculations.

Exome sequencing

Genomic DNA was purified from YUSIT1 and YUSIT1-B6 using the QIAamp DNA mini kit (Qiagen). Exome capture sequencing was performed by Perkin Elmer using the SureSelect Human All Exon 50Mb Kits to generate the library. Detection of sequencing fragments was performed on the Illumina Hiseq 2000. Germline single nucleotide polymorphisms (SNPs) were identified using the Genome Analysis Toolkit (Broad Institute). One hundred percent of the 32,682 SNPs matched between YUSIT1 and YUSIT1-B6.

RAS activation assay

Amount of activated RAS was determined using RAS Activation ELISA (Millipore). Briefly, 100 micrograms of total protein lysed in magnesium lysis buffer was added to RAF-1-RBD coated glutathione plate. Captured RAS was detected by chemiluminescence using anti-Ras antibody on the SpectraMax L (Molecular Devices). Activated RAS was reported as chemiluminescnt signal in arbitrary units (AU) after subtraction of no lysate added control.

GSK1120212 MEK1 Model Construction

Crystal structure 3EQG (1) was prepared for model building using the protein preparation wizard in Maestro (Schrodinger, LLC). After the addition of hydrogens, deletion of some waters and assignment of double bonds and H-bonds, the structure was energy-minimized using Impref and the OPLS2005 force field using a heavy atom

convergence threshold of 0.15 angstroms. GSK1120212 was positioned manually in place of the allosteric inhibitor in the original structure. The GSK1120212 MEK1 complex was energy-minimized while holding the protein fixed, using Macromodel in Maestro and the OPLS2005 force field. The acetylated anilino moiety in GSK1120212 was further refined with a conformational search using Macromodel. Finally, the ligand and non-backbone atoms up to 7 angstroms from the ligand were energy minimized using Macromodel.

Small hairpin RNA (shRNA) mediated knockdown of NRAS

A375 were transduced with lentivirus expressing shRNAs targeting NRAS (TRCN0000300442) or non-targeting control (SHC002V) from Sigma Aldrich (St. Louis, MO). Cells with stable integration of the shRNA lentivirus were selected with puromycin and single-cell clones isolated with cloning cylinders. Stably integrated clones were maintained in puromycin for subsequent experiments.

Cloning and FLAG-tagging of human neuroblastoma RAS viral oncogene homolog (NRAS) cDNA

Primers used to PCR amplify wild-type human NRAS-WT were designed based on UTR flanking regions of the published human 5' and 3' UTR sequence (NM_002524.3). The full length ORF was amplified using the primers NRASfornest (5'-ATGACTGAGTACAAACTGGTG 3') and NRASrevNEST (5'-TTACATCACCACACATGGCAATC-3'). The resulting ORF was TOPO-cloned into pCR BLUNT vector (Invitrogen, Carlsbad, CA) and double-strand sequence confirmed. This plasmid was used as template for the NRAS-WT FLAG-tag and mutagenesis reactions. 5' FLAG-tag was incorporated using the primers NRASflagtag (5'

caccATGGATTACAAGGATGACGACGATAAGGGAatgactgagtacaaactggtggtgg-3') and NRASrevNEST (5'TTACATCACCACACATGGCAATC-3'). Human bone marrow cDNA (Marathon Ready, Clontech, Mountain View, CA) was used as the template and Phusion polymerase (New England BioLabs, Ipswich, MA) was used for all PCR. The primary and nested reactions were carried out according to vendor instructions using the following parameters: 29 cycles at 98^{o}C for 10s, 55^{o}C for 30 s and 72^{o}C for 15 s per kb, followed by a final 10 minute extension at 72^{o}C, in a MJ Research PTC200 thermocycler. The FLAG-tag incorporation annealed at 59^{o}C. The resulting ORF was TOPO-cloned into pcDNA3.1D (Invitrogen) and double-strand sequence confirmed.

Mutagenesis NRAS: Q61K, Q61R, and A146T

Full length ORF of human NRAS (NM_002524.3) was prepared as above and used as template for mutagenesis reactions and FLAG-tag reactions. Pfu Ultra polymerase (Stratagene, Santa Clara, CA) was used for PCR amplification. The reaction was performed for 16 cycles at 95^{o}C for 30 s, 60^{o}C for 60s and 72^{o}C for 14 min, then held at 4^{o}C. After mutagenesis was completed, DpNI enzyme was added to each reaction and incubated at 37^{o}C before transformation. Clones were sequenced, to verify the desired mutation. Following sequence analysis, a plasmid prep was prepared and the 5' FLAG-tag was incorporated as above. The resulting ORF was TOPO-cloned into pcDNA3.1D (Invitrogen) and double strand sequence confirmed.

Mutagenesis primers:

Q61Kfor (5'-GTTTGTTGGACATACTGGATACAGCTGGAAAAGAAGAGTACAGTGCCATGAGAGACCAAT-3')

Q61Krev (5'- TTGGTCTCTCATGGCACTGTACTCTTCTTTTCCAGCTGTATCCAGTATGTCCAACAAAC-3')

Q61Rfor (5-GTTTGTTGGACATACTGGATACAGCTGGACGAGAAGAGTACAGTGCCATGAGAGACCAAT-'3)

Q61Rrev (5'- ATTGGTCTCTCATGGCACTGTACTCTTCTCGTCCAGCTGTATCCAGTATGTCCAACAAAC-')

A146Tfor (5'- GTTACGGGATTCCATTCATTGAAACCTCAACCAAGACCAGACAGGGTGTTGAAGATGCTT-'3)

A146Trev (5'- AAGCATCTTCAACACCCTGTCTGGTCTTGGTTGAGGTTTCAATGAATGGAATCCCGTAAC-3')

NRASflagTAG (5'-caccATGGATTACAAGGATGACGACGATAAGGGAatgactgagtacaaactggtggtgg-3')

Transfection of A375 cells

A375 cells were transfected with indicated NRAS constructs (generation described in above) with lipofectamine 2000 (Invitrogen). Stable single-cell transfectants were selected with 0.1 μmol/L GSK2118436 and isolated with cloning cylinders. Colonies were observed after transfection of the vector, NRAS wild-type and NRAS mutant plasmids into A375 when selected with G418. Transfected clones were maintained in RPMI 1640 medium containing 10% FBS and 0.1 μmol/L GSK2118436 and plated without GSK2118436 for proliferation and western blot experiments.

Small-Interfering RNA (siRNA) mediated knockdown of MEK1 and MEK2

Individual oligo siRNAs (Dharmacon) for MAP2K1 (D-003571-01 (MEK1#1), D-003571-04 (MEK1#4) and D-003571-05 (MEK1#5)), MAP2K2 (D-003573-01 (MEK2#1) and D-003573-02 (MEK2#2)) and non-targeting controls (D-001210-02 and D-001210-05) were reverse transfected twice into A375, 12R5-1, and 12R5-5 cells 48 hours apart using RNAiMAX (Invitrogen). Twenty-four hours after the second transfection, the cells were plated for proliferation and protein extraction. Protein was extracted 24 hours after plating. Proteins were subjected to western blot analysis. Relative growth over 72h was determined by Cell Titer-Glo®. Triplicate samples were averaged and expressed as percent of growth in the mock transfected cells.

BacMAM Plasmid construction.

The coding regions of human MEK1 and MEK2 were PCR-amplified, digested, and cloned using restriction sites EcoRI-BamHI and HindIII-HpaI, respectively. Then they were cloned into pFNcmvNA vector (2). The resultant pFNcmvNA-human MEK1 and

pFNcmvNA-human MEK2 plasmids were mutated to construct their mutants by QuickChange Lighting site-directed mutagenesis (Agilent Technologies) with primers used at the following positions:

MEK1wt		forward 5'-GAATTCGGTACCATGCCCAAGAAGAAGCCGACGCCCAT-3'	
		reverse 5'-GTTAACGGATCCTTAGACGCCAGCAGCATGGGTTGGTGTGC-3'	
MEK1Q56P		forward 5'-CGCCTTGAGGCCTTTCTTACCccgAAGCAGAAGGTGGGAGAACTG-3'	
		reverse 5'-CAGTTCTCCCACCTTCTGCTTcggGGTAAGAAAGGCCTCAAGGCG-3'	
MEK1Het59delK		forward 5'-GAGGCCTTTCTTACCCAGAAGcaggtgGGAGAACTGAAGGATGACGAC-3'	
		reverse 5'-GTCGTCATCCTTCAGTTCTCCcacctgCTTCTGGGTAAGAAAGGCCTC-3'	
MEK1P387S		forward 5'-GGCCTTAACCAGCCCAGCACAtcaACCCATGCTGCTGGCGTCtaa-3'	
		reverse 5'-ttaGACGCCAGCAGCATGGGTtgaTGTGCTGGGCTGGTTAAGGCC-3'	

BacMam generation

BacMam viruses were generated as described (2), which is based on the Bac-to-Bac system that of LifeTechnologies (Invtirogen). Briefly pFNcmvNA-human MEK1 and pFNcmvNA-human MEK2 and their mutant derivatives were transformed into DH10Bac cells. The resulting recombinant bacmid DNA was used to transfect Sf9 cells. After 3-4 days, P0 batches of BacMams were harvested, which were used to infect Sf9 cells to generate P1 batch BacMams.

BacMAM transduction of A375 cells

BacMAM constructs were generated as described above. BacMAM virus particles were prepared as described above. A375 cells were transduced with the indicated BacMAM virus. Twenty-four hours post transduction, cells were plated for subsequent proliferation and western blot analysis as described above.

Affymetrix expression analyses

Clone 16R6-4 was selected for comparison with A375 cells after compound treatment with GSK2118436 and GSK1120212 alone and in combination with each other for 24 hours. Three independent samples were used for each clone or each treatment of

clone. RNA and cDNA were prepared and microarray analyses were performed at Response Genetics Inc. (Los Angeles, CA) using Affymetrix HG-U133Plus2 array according to the standard Affymetrix protocol. RNA microarray signals were normalized by MAS5. QC and data analysis were performed using Array Studio (Omicsoft). Samples with a MAD score <-5 were excluded from further analysis. Differentially expressed genes were identified by applying one-way ANOVA with p-value <0.05 and >2-fold change. Probe sets that met the cut-off criteria, but had a mean intensity less than 100 were excluded.

Supplemental References

(1) Fischmann TO, Smith CK, Mayhood TW, Myers JE, Reichert P, Mannarino A, et al. Crystal structures of MEK1 binary and ternary complexes with nucleotides and inhibitors. Biochemistry 2009;48:2661-74.

(2.) James A. Fornwald, Quinn Lu, Dayuan Wang, and Robert S. Ames: GeneExpression in Mammalian Cells using BacMam, a Modified Baculovirus System Methods in Molecular Biology, vol. 338(95), 2007

Supplementary Table 1. Inhibition of cell growth by GSK2118436, GSK1120212, GSK2126458 and their combination in YUSIT1 clones with acquired resistance to GSK2118436

			Single Agent IC_{50} (µmol/L)			GSK2118436+GSK1120212			GSK2118436+GSK2126458			GSK1120212+GSK2116458		
CLONE	NRAS	MEK1	GSK2118436	GSK1120212	GSK2116458	IC_{50}[1]	EOHSA[2]	CI[3]	IC_{50}[4]	EOHSA	CI[3]	IC_{50}[5]	EOHSA	CI[3]
YUSIT1	WT	WT	0.014 ± 0.003	0.0007 ± 0.0002	0.031 ± 0.007	0.003 ± 0.001	22.2 ± 0.1	0.83 ± 0.21	0.009 ± 0.002	8.6 ± 5.2	0.64 ± 0.27	0.0005 ± 0.0001	8.6 ± 3.1	0.74 ± 0.06
YUSIT1-B5	Q61K	WT	>2	0.0049 ± 0.0017	0.047 ± 0.010	0.034 ± 0.006	6.6 ± 3.2	N/A[6]	0.291 ± 0.046	10.2 ± 1.7	N/A	0.0029 ± 0.0009	11.5 ± 2.0	0.68 ± 0.09
YUSIT1-B11	Q61K	WT	0.527 ± 0.146	0.0023 ± 0.0008	0.024 ± 0.008	0.019 ± 0.009	3.4 ± 2.1	0.92 ± 0.10	0.115 ± 0.056	12.4 ± 5.6	0.98 ± 0.20	0.0019 ± 0.0010	5.6 ± 1.4	0.91 ± 0.07
YUSIT1-B31	Q61K	WT	0.997 ± 0.154	0.0026 ± 0.0005	0.036 ± 0.006	0.017 ± 0.004	6.2 ± 3.3	0.80 ± 0.13	0.209 ± 0.034	10.3 ± 0.6	0.97 ± 0.09	0.0024 ± 0.0006	5.2 ± 5.6	0.91 ± 0.22

[1] IC_{50} for GSK2118436 dosed in a 10:1 molar to molar combination with GSK1120212 expressed in µmol/L

[2] EOSHA, excess over single agent expressed as percentage points

[3] CI, Combination Index

[4] IC_{50} for GSK2118436 dosed in a 10:1 molar to molar combination with GSK2126458 expressed in µmol/L

[5] IC_{50} for GSK1120212 dosed in a 1:1 molar to molar combination with GSK2126458 expressed in µmol/L

[6] N/A, not applicable

Supplementary Figure Legends

Supplementary Fig. 1S. Supplemental Figure S1. RAS activity in A375, 12R5-3 ($NRAS^{A146T}$), 12R8-1 ($NRAS^{A146T}$), and 16R6-4 ($NRAS^{Q61K,A146T}$, $MEK1^{P387S}$) as described in the supplementary methods

Supplemental Figure S2. GSK2118436 induced BRAF-CRAF heterodimer complex. A375, 12R5-3 and 16R6-4 were treated with indicated concentration of GSK2118436 for 24 hours. Protein was extracted and immunoprecipated with BRAF or CRAF antibodies. Cell lysates or immunoprecipated material was analyzed by western blot with indicated antibody.

Supplemental Figure S3. Stable shRNA-mediated knockdown of NRAS in 16R6-4 ($NRAS^{Q61K,A146T}$, $MEK1^{P387S}$) partially restores sensitivity to GSK1120212 in a 3 day cell growth assay. Stable clones with control shRNA (Control_clone#2, open circle; Control_clone#7, open square) or shRNA to NRAS (NRAS_clone#2, closed diamond; NRAS_clone#9, closed triangle) were treated with various doses of GSK1120212 for 3 days and relative cell growth compared to DMSO is shown.

Supplemental Figure S4. Expression of $NRAS^{Q61K}$ or $NRAS^{A146T}$ reduces sensitivity to GSK1120212. Relative cell growth of A375 (open circles), five A375 clones expressing FLAG-$NRAS^{Q61K}$ (closed symbols) and two A375 clones (open triangles) expressing FLAG-$NRAS^{A146T}$ after treatment with GSK1120212 for three days is shown.

Supplemental Figure S5. Western Blot to confirm knockdown of MEK1 and MEK2 with specific single siRNA. A375, 12R5-1 and 12R5-5 were transfected with indicated single oligo siRNA. Forty-eight hours post transfection, protein was collected and analyzed by western blot analysis using indicated antibodies.

Supplemental Figure S6. Representative growth curves of A375 (A) and A375 derived clones that acquired resistance to GSK2118436. 12R5-1 (B), 12R5-3 (C), 16R6-2 (D), and 16R6-4 (E) after treatment with GSK2118436 (GSK436), GSK1120212 (GSK212) or their combination in a 10:1 constant molar to molar ratio for three days.

Supplemental Figure S7. Representative growth curves relative to vehicle of A375 (A) and A375 stably transfected with $NRAS^{Q61K}$ (B, C), and $NRAS^{A146T}$ (D, E) after treatment with GSK2118436 (GSK436), GSK1120212 (GSK212), or their combination in a fixed 10:1 molar ratio for three days.

Supplemental Figure S8. Representative growth curves relative to vehicle of A375 transduced with vector (A), MEK1 wild-type (WT) (B), $MEK1^{K59del}$ (C) and $MEK1^{Q56P}$ (D) after treatment with GSK2118436 (GSK436), GSK1120212 (GSK212), or their combination in a 10:1 fixed molar ratio combination for three days.

Supplemental Figure S9. Representative growth curves of resistant clones, 12R5-3 (A), 16R6-4 (B), and 12R5-5 (C) treated with increasing concentrations of GSK1120212 in the presence of 0, 0.1, 0.3 and 1μmol/L GSK2118436 for three days. A375 treated with increasing concentrations of GSK1120212 alone is shown (dashed line, open circle) for comparison.

Fig. S1

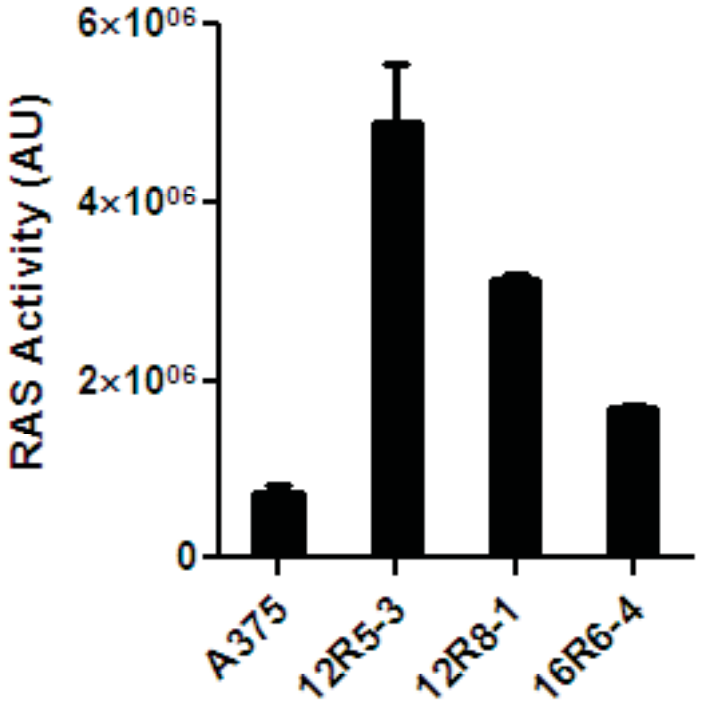


Fig. S2

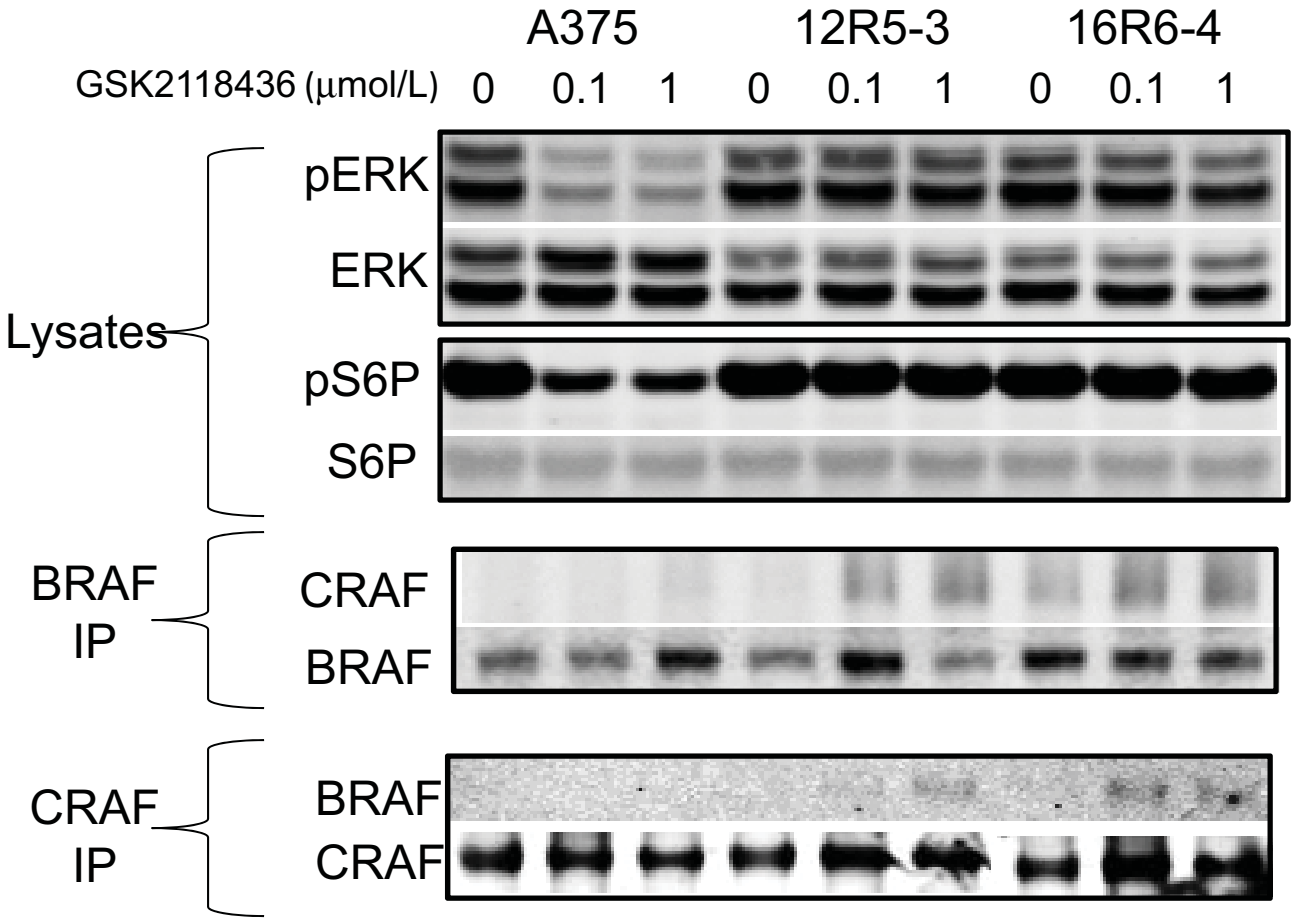

A375
12R5-3
16R6-4
GSK2118436 (μmol/L)
0
0.1
1
0
0.1
1
0
0.1
1
Lysates
pERK
ERK
pS6P
S6P
BRAF IP
CRAF
BRAF
CRAF IP
BRAF
CRAF

Fig. S3

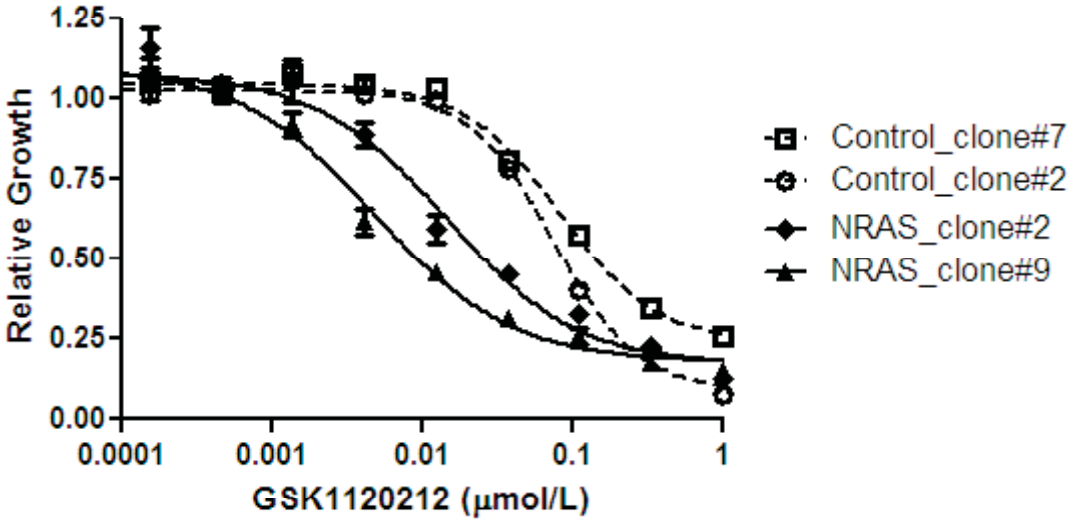

1.25
1.00
0.75
0.50
0.25
0.00
Relative Growth
0.0001
0.001
0.01
0.1
1
GSK1120212 (µmol/L)
Control_clone#7
Control_clone#2
NRAS_clone#2
NRAS_clone#9

Fig. S4

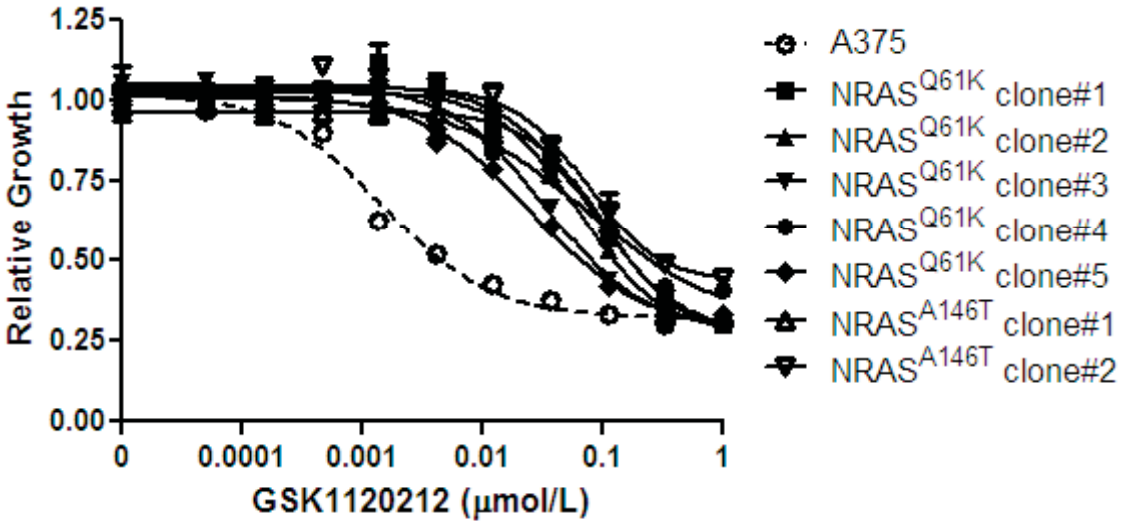

1.25
1.00
0.75
0.50
0.25
0.00
Relative Growth
0
0.0001
0.001
0.01
0.1
1
GSK1120212 (µmol/L)
A375
NRAS^{Q61K} clone#1
NRAS^{Q61K} clone#2
NRAS^{Q61K} clone#3
NRAS^{Q61K} clone#4
NRAS^{Q61K} clone#5
NRAS^{A146T} clone#1
NRAS^{A146T} clone#2

Fig. S5

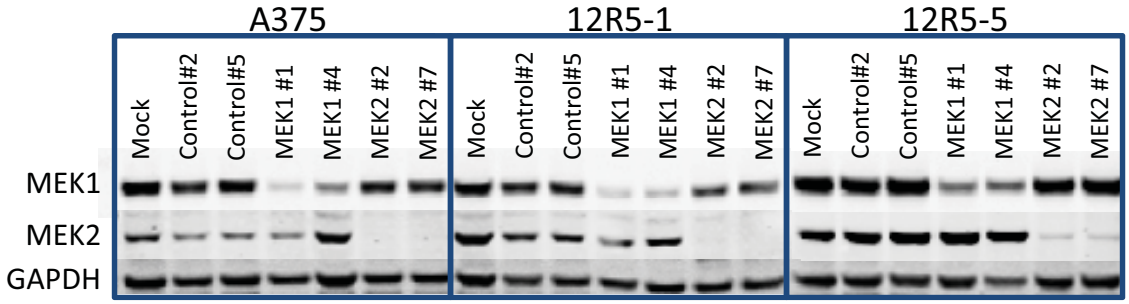

A375
12R5-1
12R5-5
Mock
Control#2
Control#5
MEK1 #1
MEK1 #4
MEK2 #2
MEK2 #7
MEK1
MEK2
GAPDH

Fig. S6

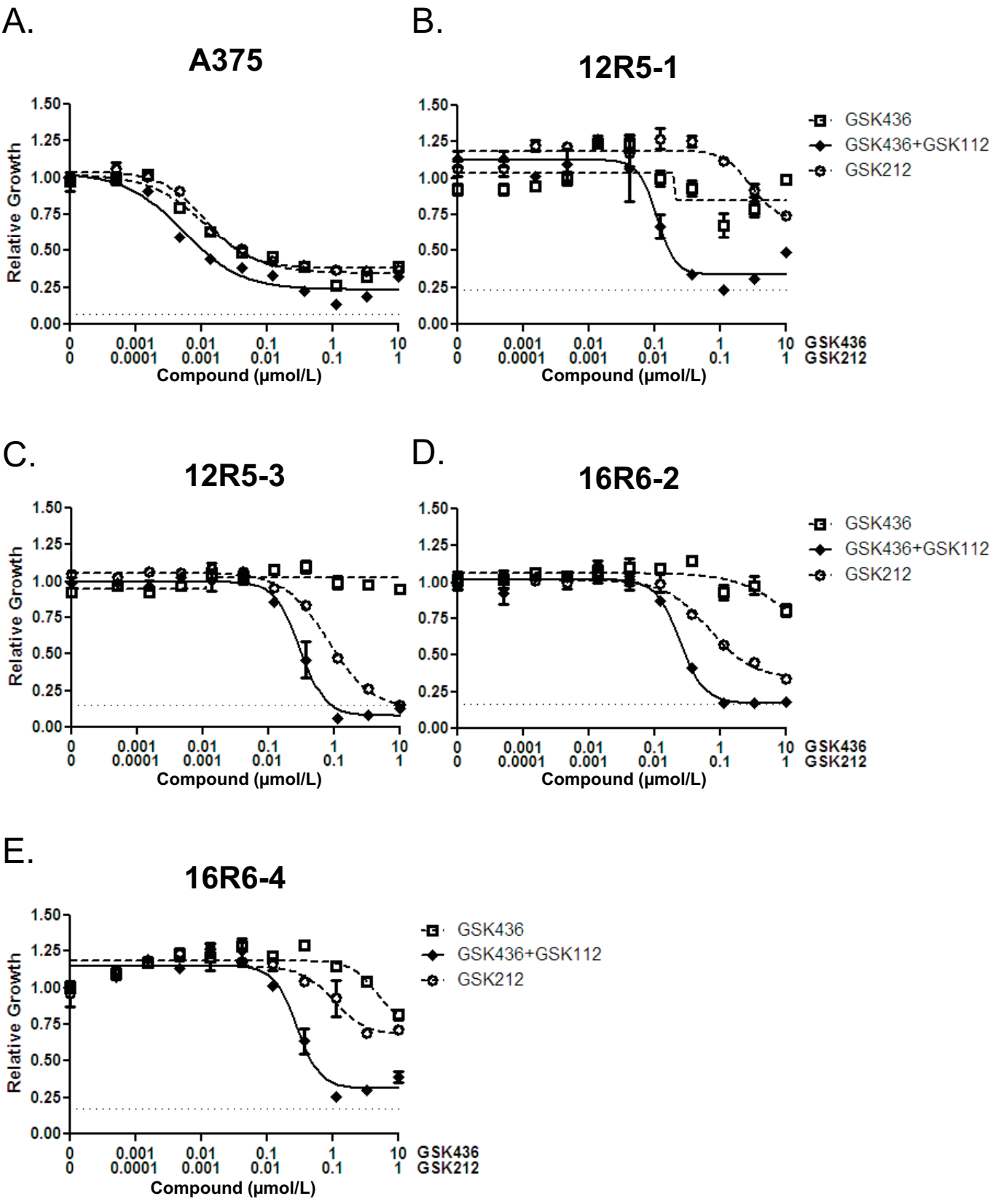

A.
A375
B.
12R5-1
C.
12R5-3
D.
16R6-2
E.
16R6-4
Relative Growth
Compound (μmol/L)
GSK436
GSK436+GSK112
GSK212
0.00
0.25
0.50
0.75
1.00
1.25
1.50
0 0.001 0.01 0.1 1 10
0 0.0001 0.001 0.01 0.1 1

Fig. S7

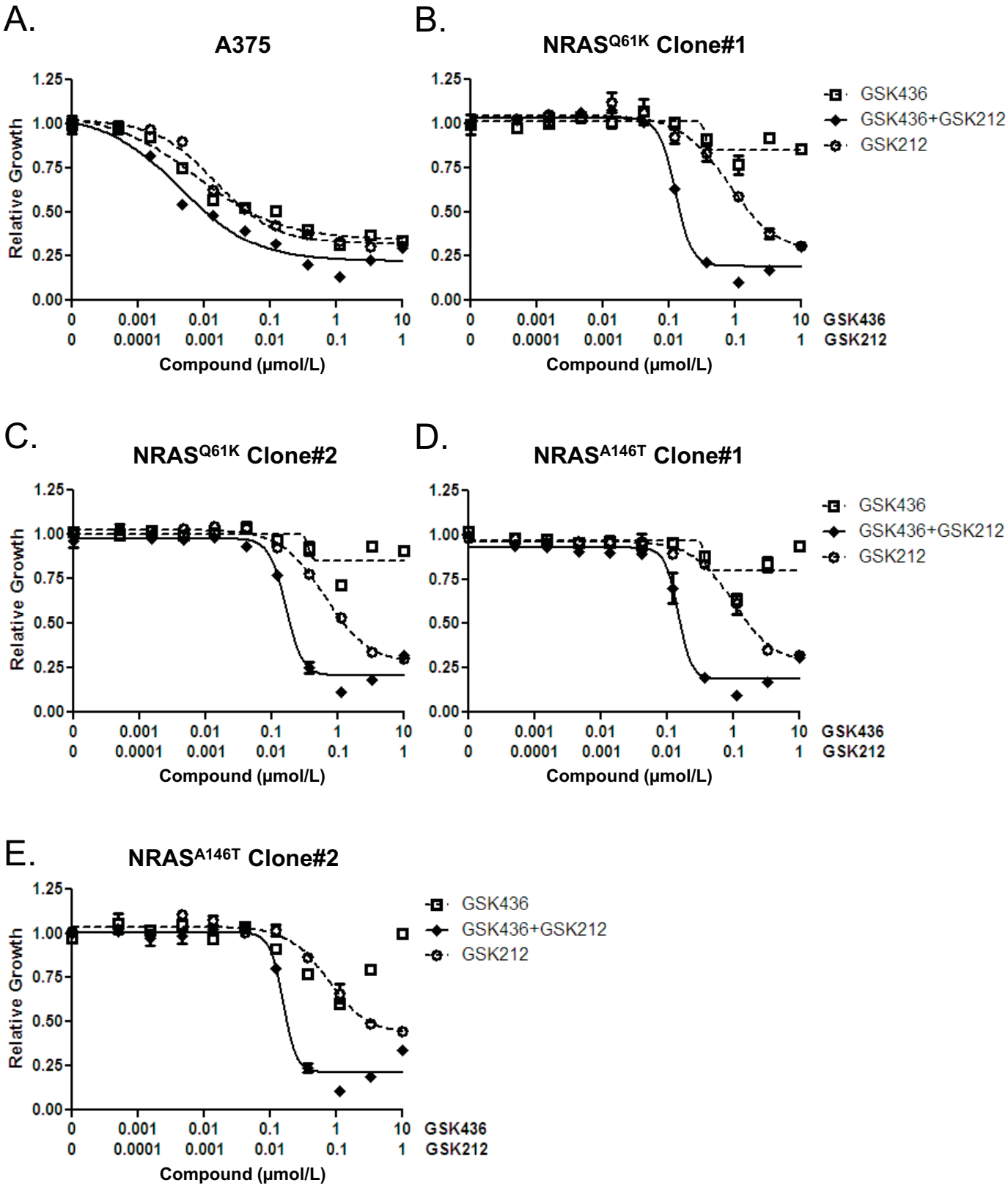

A.
A375
B.
NRASQ61K Clone#1
C.
NRASQ61K Clone#2
D.
NRASA146T Clone#1
E.
NRASA146T Clone#2
Relative Growth
1.25
1.00
0.75
0.50
0.25
0.00
0
0.001
0.01
0.1
1
10
0
0.0001
0.001
0.01
0.1
1
GSK436
GSK212
Compound (μmol/L)
GSK436
GSK436+GSK212
GSK212

Fig. S8

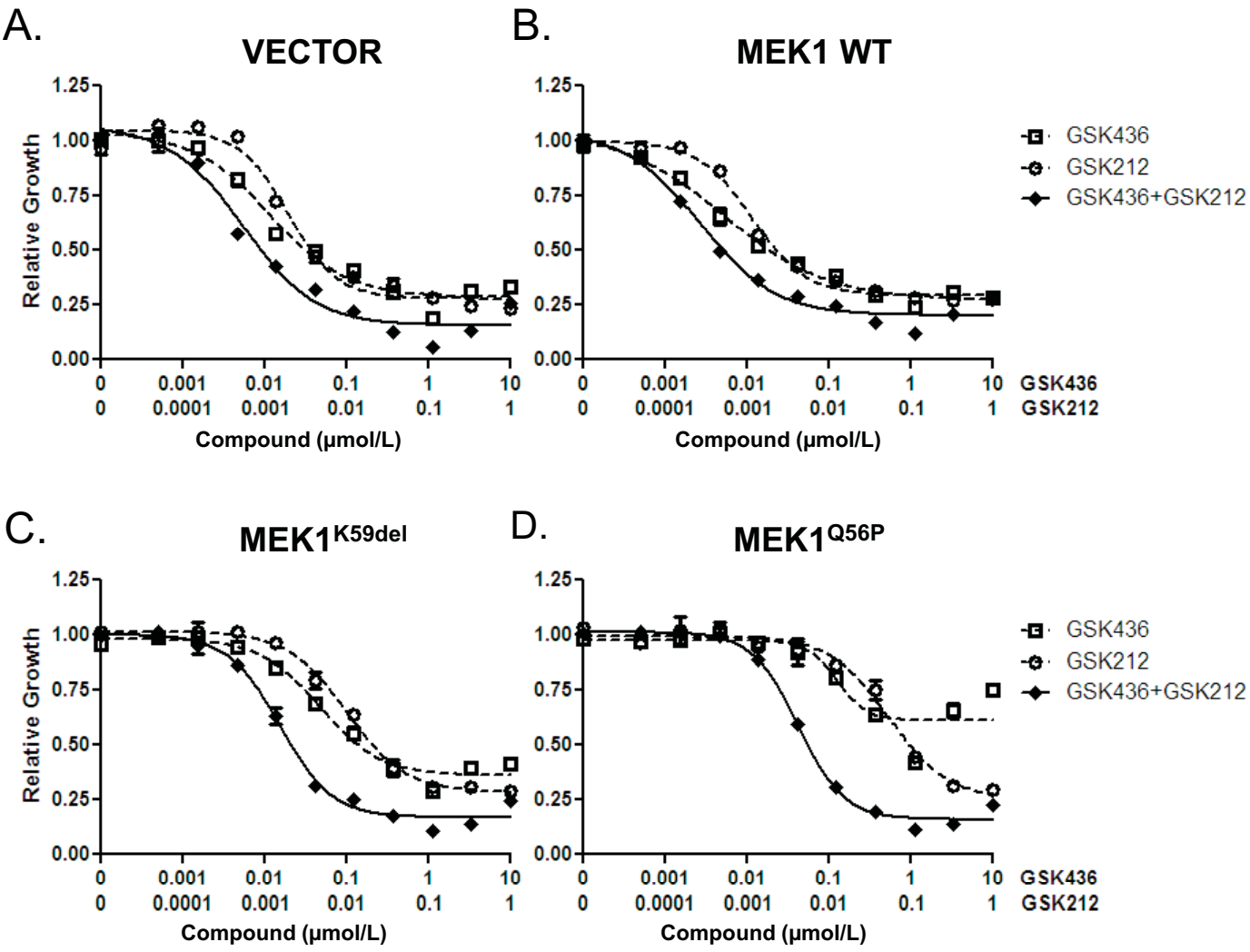
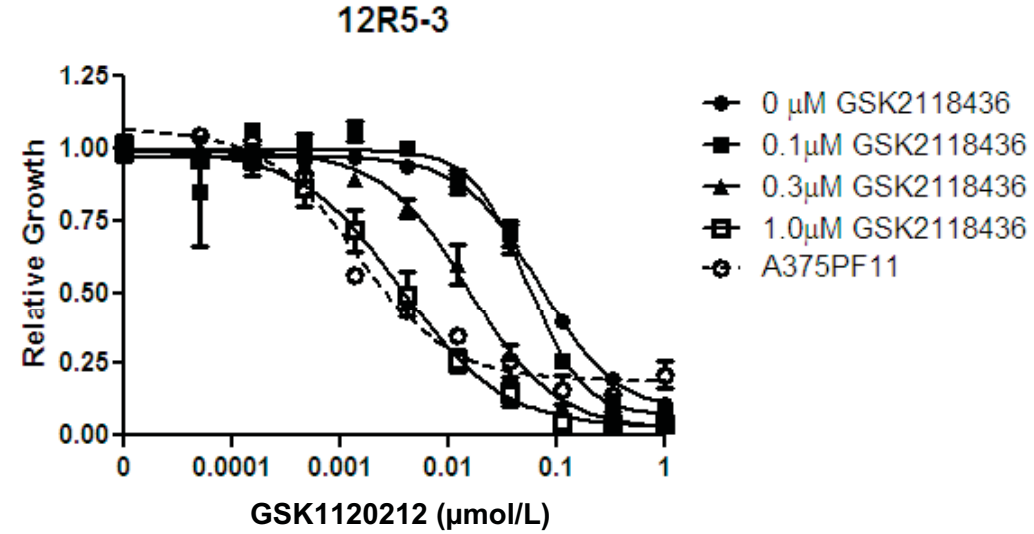
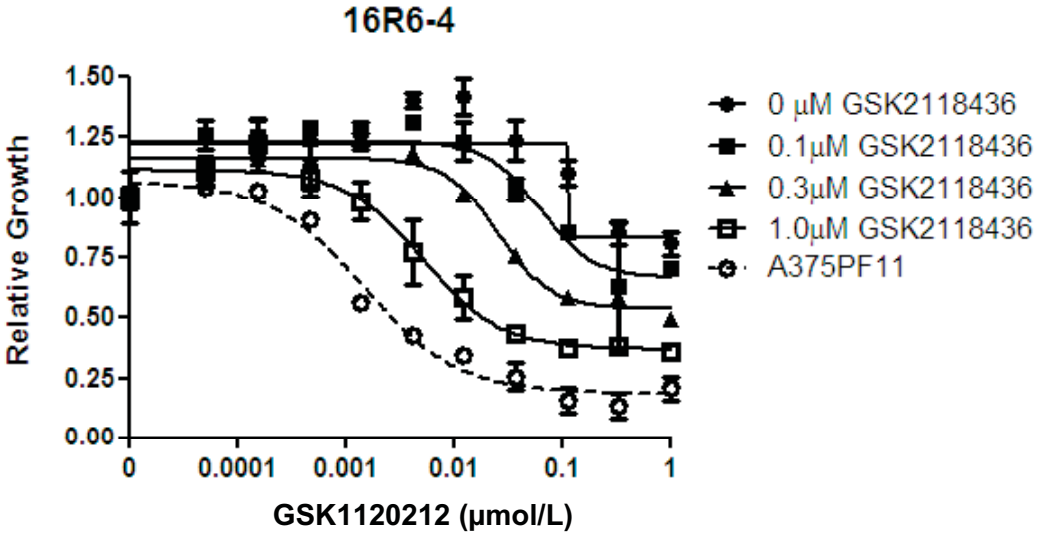

A.
VECTOR
B.
MEK1 WT
C.
MEK1K59del
D.
MEK1Q56P
Relative Growth
1.25
1.00
0.75
0.50
0.25
0.00
0 0.001 0.01 0.1 1 10
0 0.0001 0.001 0.01 0.1 1
GSK436
GSK212
Compound (µmol/L)
GSK436
GSK212
GSK436+GSK212

Fig. S9

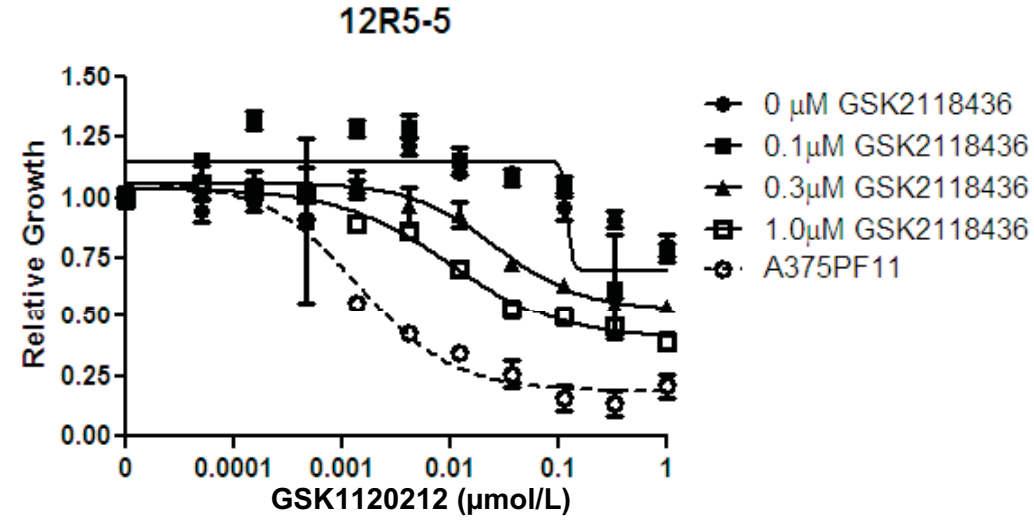
A.


12R5-3
Relative Growth
1.25
1.00
0.75
0.50
0.25
0.00
0
0.0001
0.001
0.01
0.1
1
GSK1120212 (μmol/L)
0 μM GSK2118436
0.1μM GSK2118436
0.3μM GSK2118436
1.0μM GSK2118436
A375PF11

B.


16R6-4
Relative Growth
1.50
1.25
1.00
0.75
0.50
0.25
0.00
0
0.0001
0.001
0.01
0.1
1
GSK1120212 (μmol/L)
0 μM GSK2118436
0.1μM GSK2118436
0.3μM GSK2118436
1.0μM GSK2118436
A375PF11

C.


12R5-5
Relative Growth
1.50
1.25
1.00
0.75
0.50
0.25
0.00
0
0.0001
0.001
0.01
0.1
1
GSK1120212 (μmol/L)
0 μM GSK2118436
0.1μM GSK2118436
0.3μM GSK2118436
1.0μM GSK2118436
A375PF11