# KAVRAN ET AL., SUPPLEMENTAL INFORMATION

## SUPPLEMENTAL MATERIALS AND METHODS

### Cell Culture

The human metastatic melanoma cell line, WM239A, was a kind gift of Dr. Meenhard Herlyn, Wistar Institute and cells were expanded and stored in liquid nitrogen at passage 8. These cells harbor a heterozygous *BRAF*$^{V600D}$ mutation, confirmed by RNA-seq analysis. Cells were grown in RPMI1640 media supplemented with 10% fetal bovine serum (Gemini, #100-500) and 1% penicillin/streptomycin (Gibco, #15140163) and were maintained at 37°C and 5% $CO_2$. Stable cell lines were made from WM239A cells at passage 20 and all experiments were performed within 5 passages of thawing cells. Inhibitors of BRAF$^{V600E}$ (LGX818/encorafenib, #S7108) and MKK1/2 (MEK162/binimetinib, #S7007) were from Selleck Chemicals. Long term cell cultures were seeded in 96-well plates at 500 cells per well or in 10 cm dishes at 86,000 cells per dish and growth media with or without drug were refreshed every Monday, Wednesday and Friday. Cell viability was measured using the CellTiter-Glo 2.0 Assay (Promega, #G9242) according to the manufacturer's protocol and calibrated against a standard curve of cells seeded at 2-fold dilutions between 125-64,000 cells per well. For dose response measurements, cells were seeded in 96-well plates at 2,500 cells per well and cell viability data were collected 72 h afterwards.

### Plasmids and Cell Lines

Plasmids for expression of each resistance oncogene were constructed by Boulder Genetics. Briefly, open reading frames for human *BRAF*$^{V600E}$, *p61-BRAF*$^{V600E}$, *MKK2*$^{C125S}$, *EGFR*$^{L858R}$, and *NRAS*$^{Q61K}$ were inserted into an inducible PiggyBac vector (Systems Biosciences, #PBQM800A-1). Plasmids and PiggyBac transposase (Systems Biosciences, #PB210PA-1) were electroporated into WM239A cells using the Neon transfection system (Invitrogen, #MPK10096) with 2 pulses x 20 ms pulse width at 1200 V. Stably transfected cells were selected for by addition of 1 μg/mL puromycin to growth media for 7 days. Resistance genes were induced with 30 μg/mL cumate (Systems Biosciences, #QM150A-1) for 72 h, followed by treatment with LGX818 or MEK162.

### Immunoblotting/Gel Shift Assays

Cells were lysed in RIPA buffer [10 mM Tris pH 8.0, 140 mM NaCl, 1% (v/v) Triton X100, 0.1% (w/v) SDS, 1 mM EDTA, 0.25% (w/v) sodium deoxycholate, 1 mM NaF, 1X EDTA-free protease inhibitors (Roche, #11873580001) and 1X PhosStop phosphatase inhibitors (Roche, #4906845001)] and sonicated at low power using a needle homogenizer. Protein concentrations were determined using the DC protein assay (Bio-Rad, #5000111). Protein samples (10 μg/lane) were separated on Tris-glycine gels or 4-20% protean-TGX precast gradient gels (Bio-Rad, #4561094) and proteins were transferred to PVDF-SQ membranes (Millipore, #ISEQ00010) for 1 h at 100 V. Membranes were incubated in primary antibody for 2 h at room temperature or overnight at 4 °C and in secondary antibody for 1 h at room temperature. Low-bis SDS-PAGE gels (10% acrylamide/0.13% bis-acrylamide) were used to separate unphosphorylated and phosphorylated forms of ERK1/2 (28). Antibodies for immunoblotting were from Cell Signaling Technology unless otherwise noted: ERK2 (#4696), phospho-p44/p42 ERK1/2-pThr202/pTyr204 (#4370), MKK1/2 (#9122), phospho-MKK1/2-pSer217/pSer221 (#9121), EGFR (#4267), pan-Ras (Millipore, #OP40), BRAF (Santa Cruz Biotechnology, #sc-166), GFP (Santa Cruz Biotechnology, #sc-9996), mCherry (GeneTex, #GTX59788), GAPDH (#5174), phospho-RSK (#9335), and beta-tubulin (#2146).

**Propidium Iodide Assay for Cell Death**

Cells were collected for flow cytometry by trypsinization and resuspension in PBS. In order to assess cell viability, cells grown in 10 cm plates were trypsinized from the plate and collected together with cells floating in media by centrifugation. Cells were then resuspended in PBS + 2 μg/mL propidium iodide (Sigma-Aldrich, #P4170), incubated for 20 min, and analyzed on a FACSCelesta flow cytometer (BD Biosciences). Flow cytometry was also used to quantify oncogene expression by monitoring GFP, CFP or mCherry. For long term experiments, GFP calibration beads (Takara Bio, #632594) were used to confirm consistency of the GFP signal over successive weeks. Flow cytometry data analysis was performed using Flowing Software 2 (http://flowingsoftware.btk.fi/) and FCSalyzer (https://sourceforge.net/projects/fcsalyzer/).

**Flow cytometry analysis of L1CAM protein expression**

Cells grown in 10 cm dishes were washed with twice with PBS and harvested by incubation in 0.5 mM EDTA in PBS at room temperature. Cells were centrifuged and resuspended in 50 μL PBS. Then the cells were fixed by diluting 4% PFA in PBS to the tube for a final concentration of 2% PFA. This incubated at 37°C for 5 minutes, and the PFA was quenched by adding 2.5 M glycine in PBS for a final concentration of 125mM. The cells were spun down at 300 rcf and washed once with 125 mM glycine in PBS. The cells were resuspended in ice cold 0.5% BSA in PBS and stored at 4°C. Once all the samples were collected, 150,000 cells per sample were aliquoted into tubes, centrifuged, and resuspended in 100 uL of 0.5% BSA in PBS and 2.5 μL of either L1CAM antibody conjugated to BV421 fluorescent dye (BD Horizon cat# 565733) or isotype control (BD Horizon # 562439). Cells were mixed by gentle vortexing and incubated in the dark on an orbital shaker for 1 hour at room temperature. The cells were washed twice and then resuspended in 0.5% BSA in PBS and analyzed on a FACSCelesta flow cytometer (BD Biosceinces). Data analysis was performed using custom scripts in R (v.4.0.3).

**Gene Expression Profiling**

Cells were rinsed with PBS, trypsinized, and centrifuged for 5 min at 300xg, then washed with 1 mL PBS and centrifuged again. After aspirating away PBS, cells were immediately frozen in liquid nitrogen and stored at -80ºC. At the end of the timecourse, all samples were thawed on ice and total RNA was purified using the Quick-RNA microprep kit (Zymo Research, #R1050) according to the manufacturer's protocol. RNA-seq was conducted by the Functional Genomics Shared Resource at the University of Colorado Cancer Center, Aurora CO. RNA quality was assessed using a 4200 TapeStation System (Agilent Technologies) and 100 ng RNA was used to prepare mRNA libraries using the Universal Plus mRNA-seq kit (NuGEN). Sequencing was performed on a HiSeq4000 System (Illumina) with 150 bp reads and 30 million reads per sample.

Adapter sequences and low quality base reads were removed using Trimmomatic (v.0.32, LEADING:3 TRAILING:3 SLIDINGWINDOW:4:15 MINLEN:36) (1). Reads were then mapped to the *Homo sapiens* genome (GRCh38) using the alignment software, HISAT2 (v.2.0.3-beta, –dta –rna-strandness F) (2). The resulting SAM file was converted to a sorted BAM file using SAMtools (v.0.1.18) (3). Reads that mapped to genes in the GRCh38.84.gtf annotation file were counted using the htseq-count script in HTSeq (v.0.6.1, -f bam -r pos -s yes -t exon -m union -i gene_id) (4). All downstream analyses were performed using R (v.4.0.3) and R Studio (v. 1.3.1093). Data were pre-filtered for genes that had at least 60 reads. The DESeq2 package (v.1.30.1) was used to normalize samples by library size and estimate dispersion factors (5). For visualizations and statistical learning methods, counts were $\log_2$ transformed using the *vst*

(variance stabilizing transform log2) function in DESeq2. Principal component analysis was performed using the *prcomp* function on the *vst* transformed data that was mean centered and set to unit variance for the 6000 genes with the highest variance across the 29 samples. The top 400 genes with the most negative or positive loadings per principal component were assigned to that PC for visualization in a heatmap.

**Analysis of melanoma cell lines in cancer cell line encyclopedia (CCLE)**

RNA-seq unnormalized count data (version 18Q1) and drug sensitivity data (NP24.3009) were downloaded from the cancer cell line encyclopedia (CCLE) website. There were 34 $BRAF^{V600}$ mutant melanoma cell lines with both RNA-seq data and $IC_{50}$ data for PLX4720. The RNA-seq data for these cell lines was normalized to library size using DESeq2, and $log_2$ transformed using the *vst* function, and each gene was mean centered and scaled to unit variance. This dataset was filtered for the 800 genes corresponding to PC1 in our dataset (Dataset S3), and the cell lines were clustered by hierarchical clustering.

## SUPPLEMENTAL REFERENCES


1. Bolger AM, Lohse M, Usadel B. Trimmomatic: A flexible trimmer for Illumina sequence data. Bioinformatics. 2014;30:2114–20.
2. Kim D, Langmead B, Salzberg SL. HISAT: A fast spliced aligner with low memory requirements. Nat Methods. 2015;12:357–60.
3. Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, Homer N, et al. The Sequence Alignment/Map format and SAMtools. Bioinformatics. 2009;25:2078–9.
4. Anders S, Pyl PT, Huber W. HTSeq-A Python framework to work with high-throughput sequencing data. Bioinformatics. 2015;31:166–9.
5. Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol. 2014;15:1–21.

Figure S1

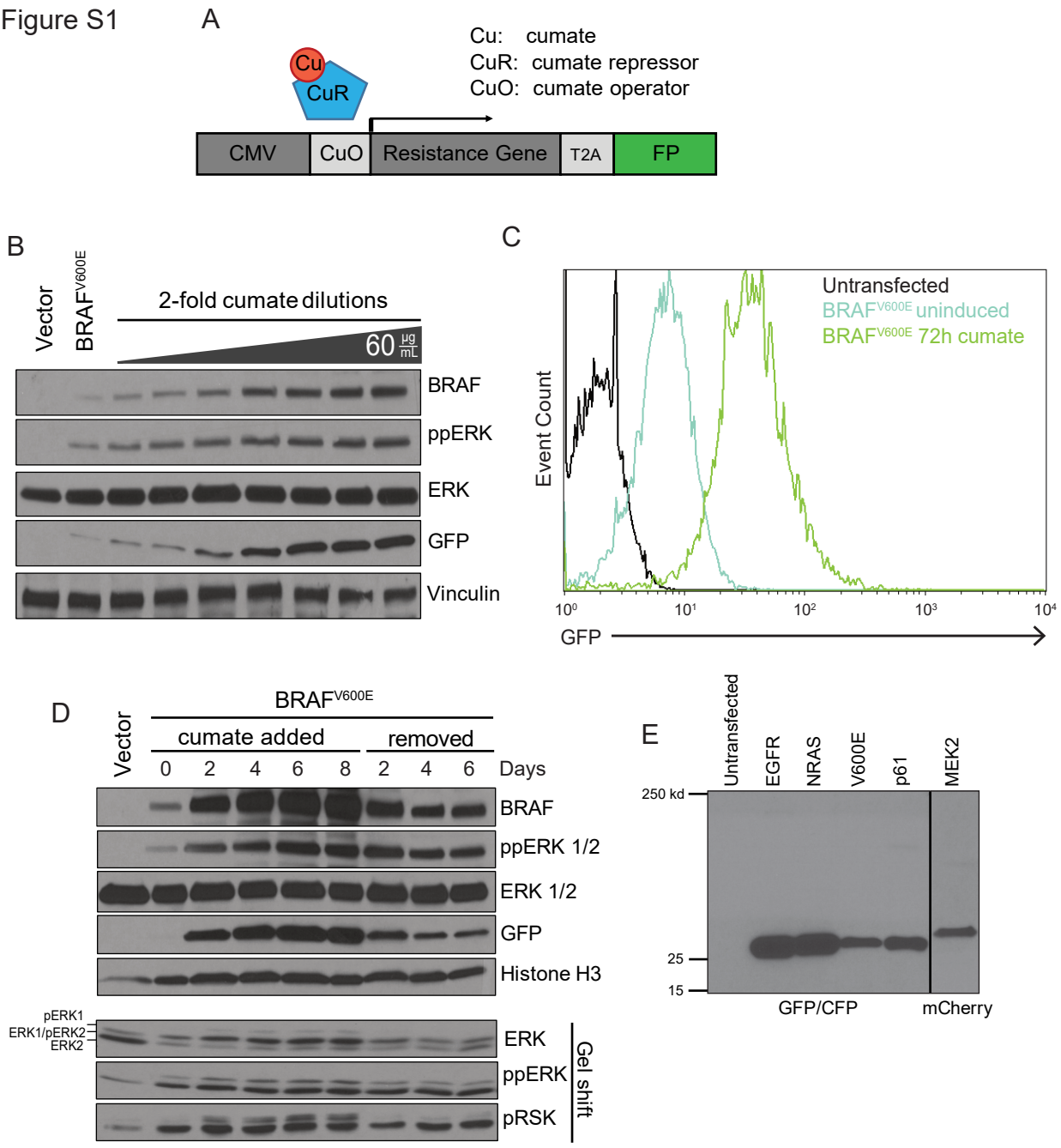


**Figure S1. Design of inducible oncogene expression vectors and characterization of expression.** **(A)** Oncogenes were expressed from stably integrated cumate-inducible PiggyBac vectors as fusion proteins with fluorescent proteins (FP) separated by a self-cleaving T2A sequence. **(B)** WM239A cells stably transfected with vectors for expression of BRAF$^{V600E}$ were induced for 72 h with serial 2-fold dilutions of cumate, starting with a maximal concentration of 60 μg/mL. **(C)** Flow cytometry analysis of GFP as a reporter for BRAF$^{V600E}$ expression, before or after cumate for 72 h. **(D)** Cells with the BRAF$^{V600E}$ vector were induced with 30 μg/mL cumate for up to 8 days, after which cumate was removed and cells were cultured in absence of cumate for up to 6 days. Cells were collected on the indicated days with or without cumate. **(E)** Immunoblot showing a single band corresponding to the cleaved FP in each cell line, confirming complete cleavage of the fusion protein after induction.

Figure S2

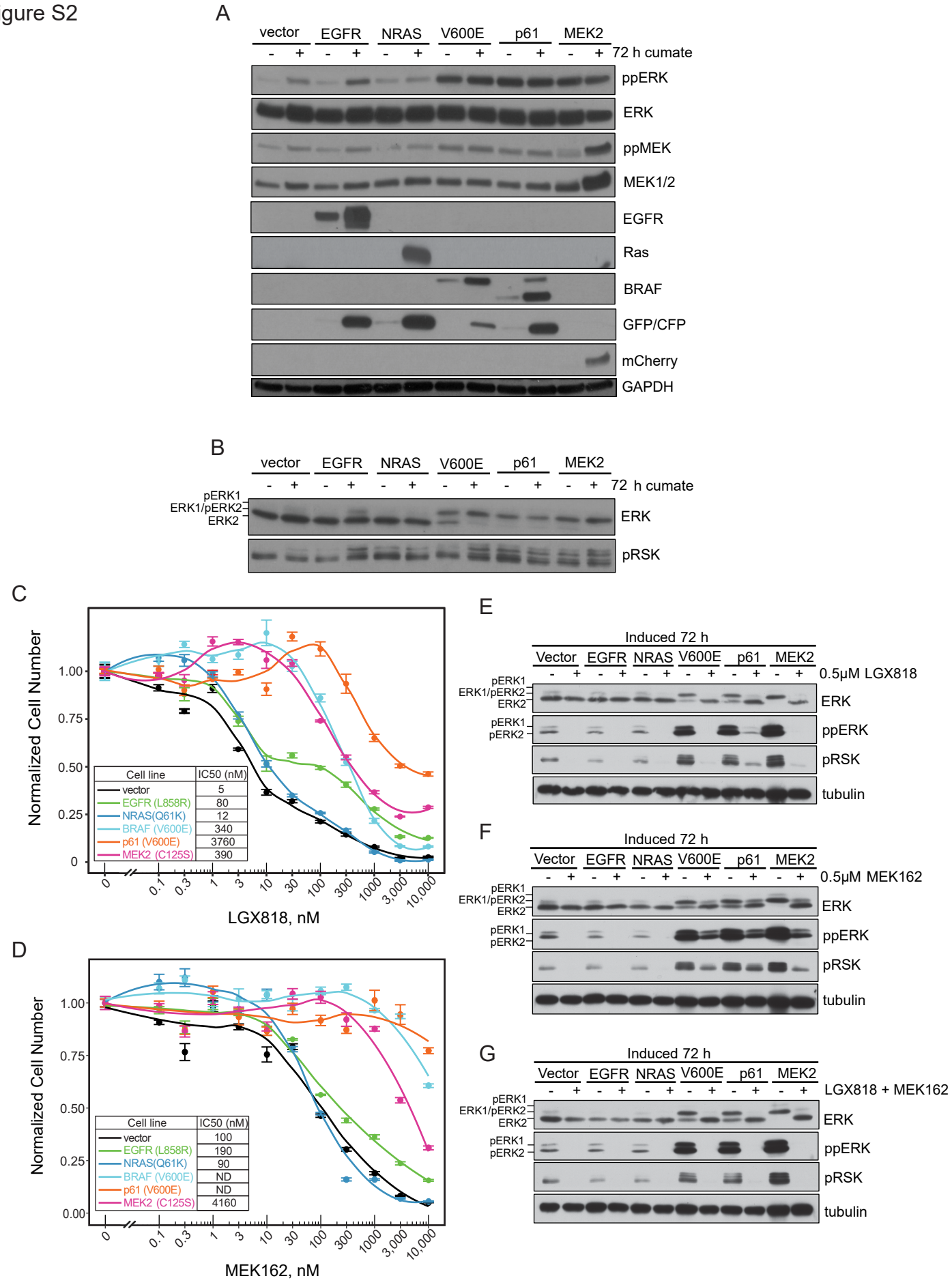


**Figure S2.** Caption continued on next page.

**Figure S2. Effects of oncogene expression on drug tolerance and ppERK signaling. (A)** WM239A cells with integrated constructs of different oncogenes were untreated or cumate-induced for 72 h prior to harvesting lysates for Western blotting of the indicated proteins. The higher signal from ppERK in lane 2 compared to lane 1 is not reproducible and is ascribed to higher total ERK in this experiment. **(B)** The same lysates were separated by low-bisacrylamide SDS-PAGE to resolve different phosphoforms of ERK1/2 and phosphorylated p90RSK (pS380). **(C)** WM239A cells were cumate-induced for 72 h, then seeded in 96 wells and treated with varying amounts of LGX818 (n = 4). **(D)** Cells were induced as in (C) but treated with MEK162 for 72 h (n = 4). **(E-G)** WM239A cells were cumate-induced for 72 h and then treated for 2 h with **(E)** 500 nM LGX818, **(F)** 500 nM MEK162, or **(G)** 500 nM LGX818 + 500 nM MEK162. Lysates were separated by low-bisacrylamide SDS-PAGE to monitor phosphorylation of ERK (pTpY) and p90RSK(pS380).

Figure S3

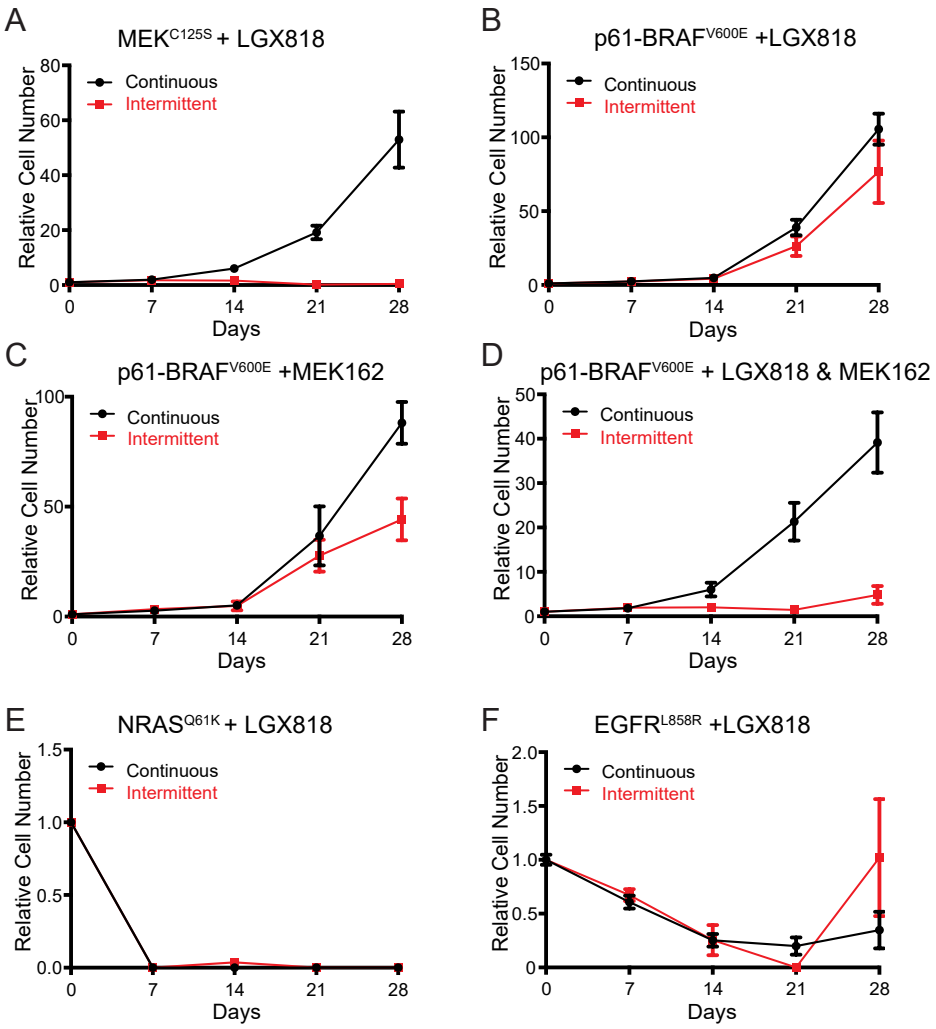


**Figure S3. Growth curves of WM239A cells engineered to express different inducible oncogenes.** WM239A cells were cumate-induced for 72 h, seeded in 96 wells, and cultured for 4 weeks under continuous or intermittent treatment conditions. The intermittent treatment cycle was 7 days on drug and 7 days off drug. Cells individually expressed **(A)** MEK2$^{C125S}$, **(B-D)** BRAF-p61$^{V600E}$, **(E)** NRAS$^{Q61K}$, or **(F)** EGFR$^{L858R}$. The inhibitors used were **(A, B, E, F)** 500 nM LGX818, **(C)** 500 nM MEK162, or **(D)** 500 nM LGX818 + 500 nM MEK162. Controls using cells transfected with empty vector are shown in Figure 2C.

Figure S4

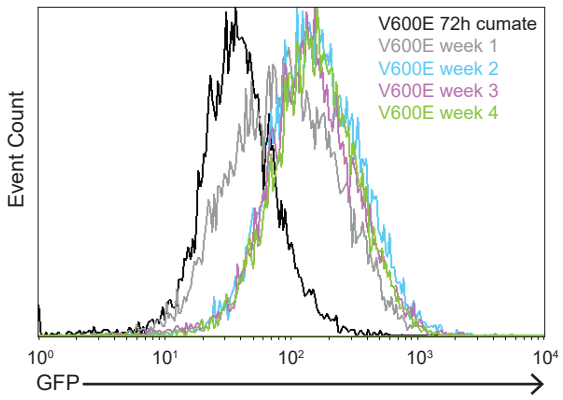


**Figure S4**. **Continuous treatment with LGX818 enriches cells with elevated expression of BRAF$^{V600E}$.** Cells were treated with 30 μg/mL cumate for 72 h to induce expression of BRAF$^{V600E}$, then cultured with 500 nM LGX818 for 4 weeks. Cells were collected at the end of each week and analyzed by flow cytometry using GFP as a reporter for BRAF$^{V600E}$ expression.

Figure S5

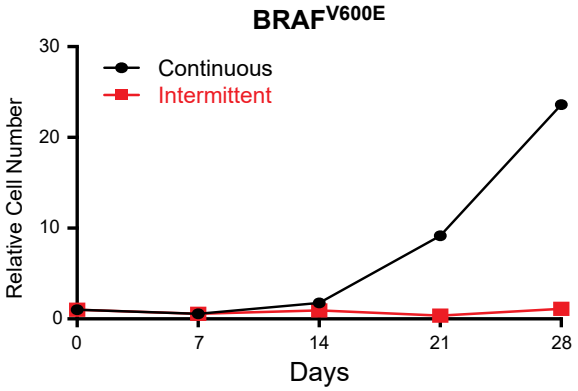


**Figure S5. Growth curves of WM239A-BRAF$^{V600E}$ cells during the RNA-seq experiment.** Cells were plated in 10 cm dishes in parallel with cells harvested for RNA-seq (Figure 4), quantifying cell numbers for continuous and intermittent RNA-seq experiments using the Cell Titer-Glo assay (n=1).

Figure S6

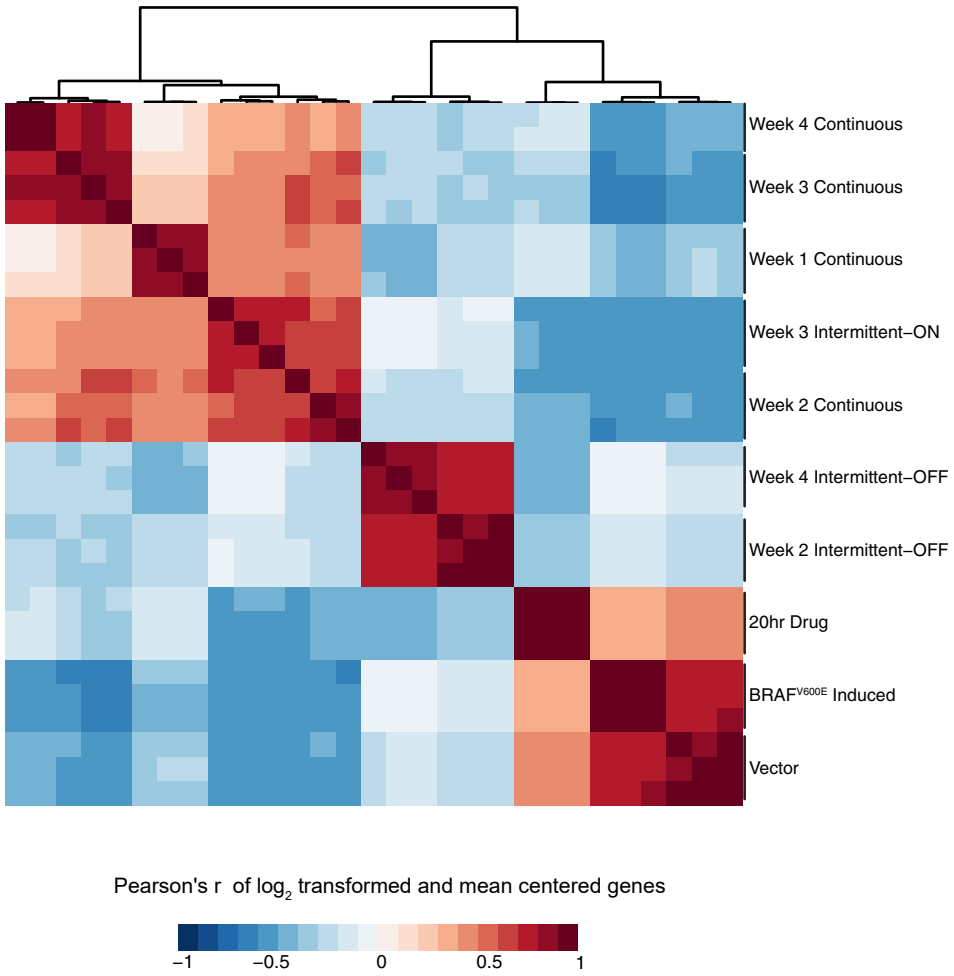


**Figure S6. Correlation matrix of RNA-seq datasets.** The normalized counts for each sample were $\log_2$ transformed and mean-centered per gene. Pearson's correlation coefficient r was calculated for all pairwise comparisons of samples. The matrix was clustered using hierarchical clustering.