| Cell Line | Vemurafenib $GI_{50}$ (μM) | Selumetinib $GI_{50}$ (nM) |
|---|---|---|
| YUMAC | 1.03 | 22.86 |
| YUSIT1 | 0.30 | 9.77 |
| YURIF | 0.27 | 35.16 |

| Cell Line | (-) VemSel BID-1870 $GI_{50}$ (μM) | (+) VemSel BID-1870 $GI_{50}$ (μM) | (-) VemSel BRD7389 $GI_{50}$ (nM) | (+) VemSel BRD7389 $GI_{50}$ (nM) |
|---|---|---|---|---|
| YUMAC | 2.5 | Undefined | 0.5 | Undefined |
| YURIF | 7.7 | Undefined | 0.63 | Undefined |
| YUMACdr | 7.9 | 8.5 | 7.9 | 10.4 |
| YURIFdr | 3.9 | 5.9 | 0.9 | 0.5 |

Table S1: $GI_{50}$ concentrations for vemurafenib and selumetinib for three parental patient-derived xenograft lines: YUMAC, YURIF, and YUSIT. $GI_{50}$ concentrations also calculated for BI-D1870 and BRD7389 with and without combination 5μM vemnurafenib and 150nM selumetinib treatment for parental YUMAC and YURIF lines, as well as vemurafenib- and selumetinib-resistant YUMACdr and YURIFdr lines. Driver mutation for each line is a BRAF V600 mutation.

| Protein | Pearson | p value |
|---|---|---|
| STAT3 p-Y705 | 1.0 | 0.000171 |
| RBM15 | 0.9 | 0.003579 |
| ARAF | 0.9 | 0.004813 |
| Porin | 0.9 | 0.006959 |
| ER alpha p-S118 | 0.9 | 0.011285 |
| PARP (cleaved) | 0.9 | 0.013575 |
| SNAIL | 0.8 | 0.016077 |
| PARP1 | 0.8 | 0.019067 |
| JNK p-T183 p-Y185 | 0.8 | 0.025708 |
| XRCC1 | 0.8 | 0.028345 |
| c-JUN p-S73 | 0.8 | 0.031171 |
| NDRG1 p-T346 | 0.8 | 0.036241 |
| SMAD1 | 0.8 | 0.038349 |
| EGFR p-Y1068 | 0.8 | 0.04057 |
| ADAR1 | 0.8 | 0.042663 |
| SETD2 | 0.8 | 0.043182 |
| HER2 p-Y1248 | 0.8 | 0.04772 |
| RAD50 | 0.7 | 0.077815 |
| ARHI | 0.7 | 0.077852 |
| TAZ | 0.7 | 0.094244 |
| UGT1A | 0.7 | 0.108245 |
| HER3 p-Y1289 | 0.6 | 0.115707 |
| VEGFR2 | 0.6 | 0.121077 |
| SMAD4 | 0.6 | 0.127021 |
| P16-INK4A | 0.6 | 0.133265 |
| CRAF p-S338 | 0.6 | 0.140327 |
| PKCalpha p-S657 | 0.6 | 0.141832 |
| MDM2 p-S166 | 0.6 | 0.148672 |
| FN1 | 0.6 | 0.154396 |
| BAP1 | 0.6 | 0.1622 |
| STAT5A | -0.4 | 0.319697 |
| IRS1 | -0.4 | 0.316206 |
| Cyclin D1 | -0.4 | 0.315524 |
| X14-3-3beta | -0.5 | 0.295488 |
| TTF1 | -0.5 | 0.276558 |
| HIAP | -0.5 | 0.235859 |
| PI3K p85 | -0.6 | 0.196009 |
| GSK3alpha/beta | -0.6 | 0.189473 |
| MEK2 | -0.6 | 0.179342 |
| BCL2 | -0.6 | 0.178915 |
| BCL-XL | -0.6 | 0.17331 |
| PMS2 | -0.6 | 0.155452 |
| RAB11 | -0.6 | 0.14319 |
| ACC p-S79 | -0.6 | 0.13428 |
| p38 | -0.6 | 0.122475 |
| TSC1 | -0.7 | 0.109883 |

| | | |
|---|---|---|
| GAB2 | -0.7 | 0.10427 |
| MSH6 | -0.7 | 0.093132 |
| IGF1Rbeta | -0.7 | 0.086342 |
| DJ1 | -0.7 | 0.083631 |
| MEK1 p-S217 p-S221 | -0.7 | 0.077161 |
| BRAF p-S445 | -0.7 | 0.075509 |
| BRAF | -0.7 | 0.072931 |
| X14-3-3zeta | -0.7 | 0.061851 |
| FAK | -0.7 | 0.059239 |
| Stathmin 1 | -0.8 | 0.048358 |
| ETS-1 | -0.8 | 0.046985 |
| TIGAR | -0.8 | 0.043098 |
| RSK 1/2/3 | -0.9 | 0.008969 |
| Myosin | -0.9 | 0.007348 |

Table S2: Full list of top 60 most significant proteins screened by RPPA with spearman r and p values for BI-D1870 GI50 correlation.

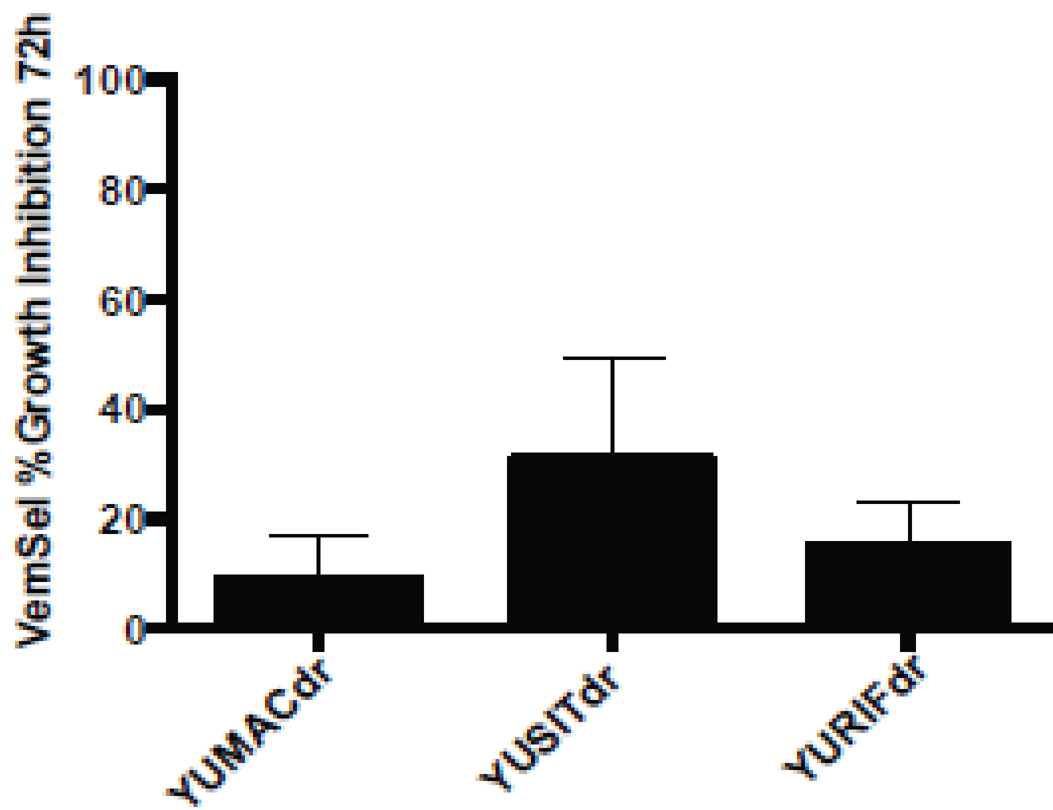



Figure S1: Growth inhibition at 72 hours of treatment with 3μM veurafenib and 150nm selumetinib for three dual-resistant melanoma cell lines.

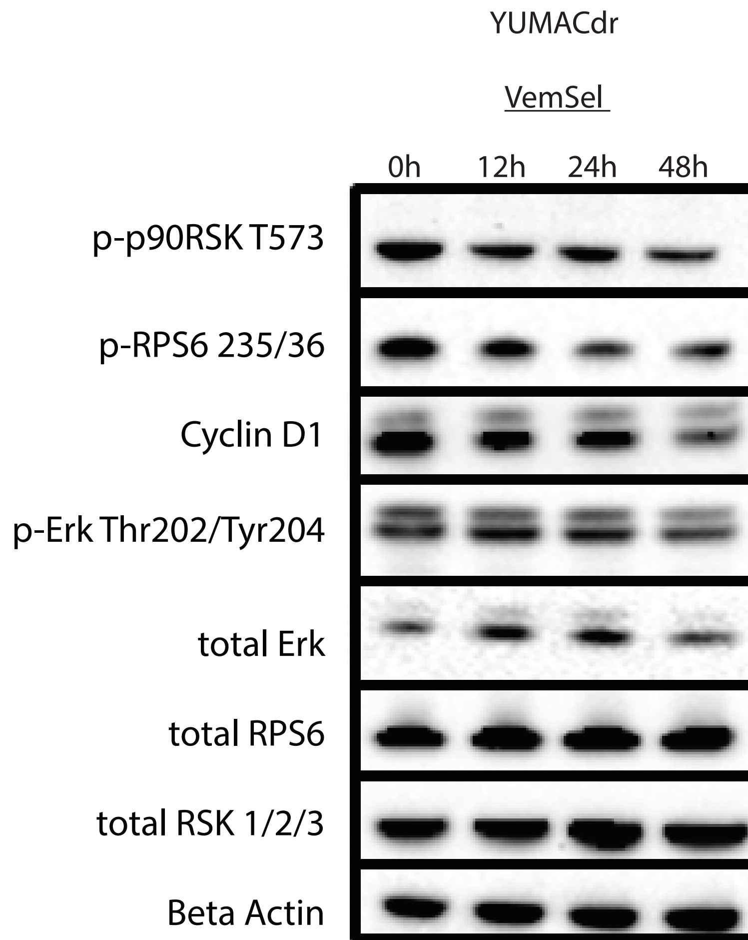



Figure S2: Treatment of YUMACdr with combination 5μM vemurafenib + 150nM selumetinib does not induce significant reduction in proliferation or control nodes for translation over time through 48 hours.

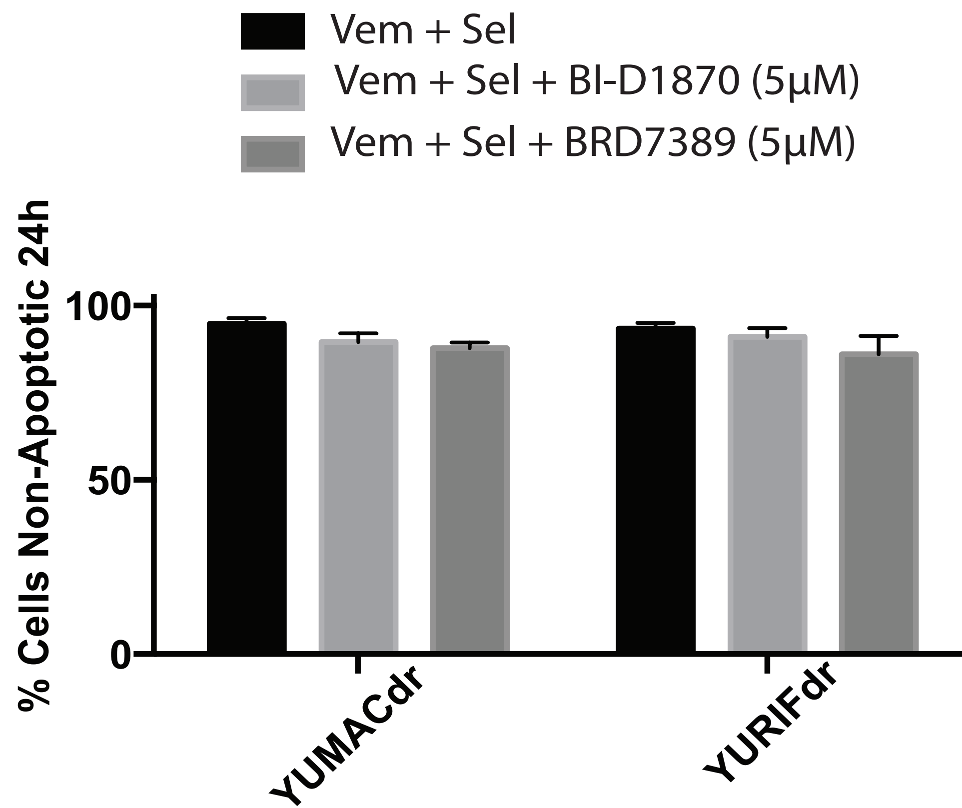



Figure S3: p90RSK inhibition causes minimal cell death after 24 hours of treatment. +/- SEM, N=3.

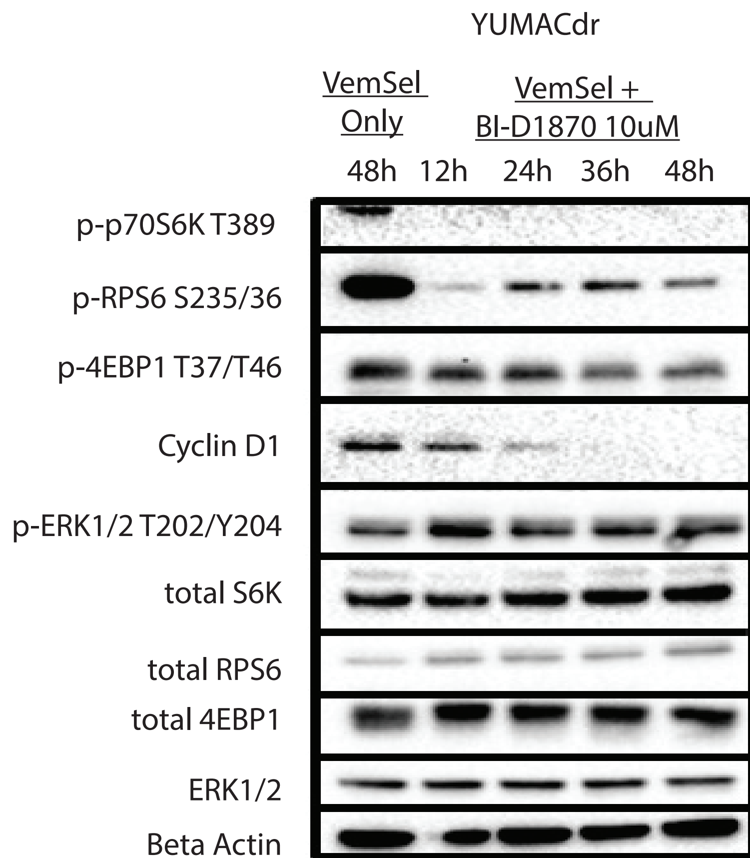



Figure S4: Treatment with 10μM BI-D1870 + 5μM vemurafenib + 150nM selumetinib induced rapid decrease in activity in control nodes for translation over vemurafenib and selumetinib alone.

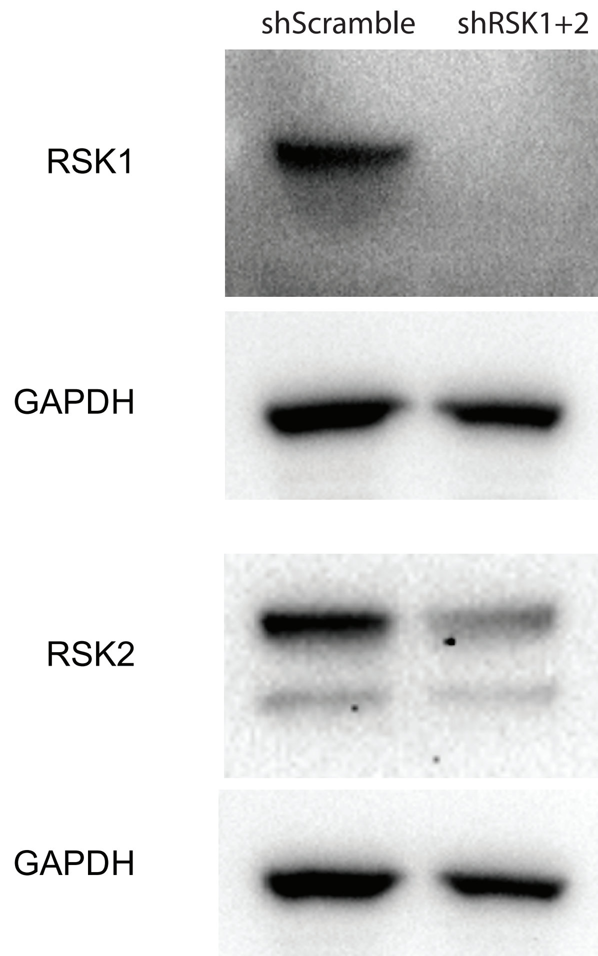



Figure S5: Western blot confirmation of knockdown of RSK1 and RSK2 by adenoviral shRNA infection.

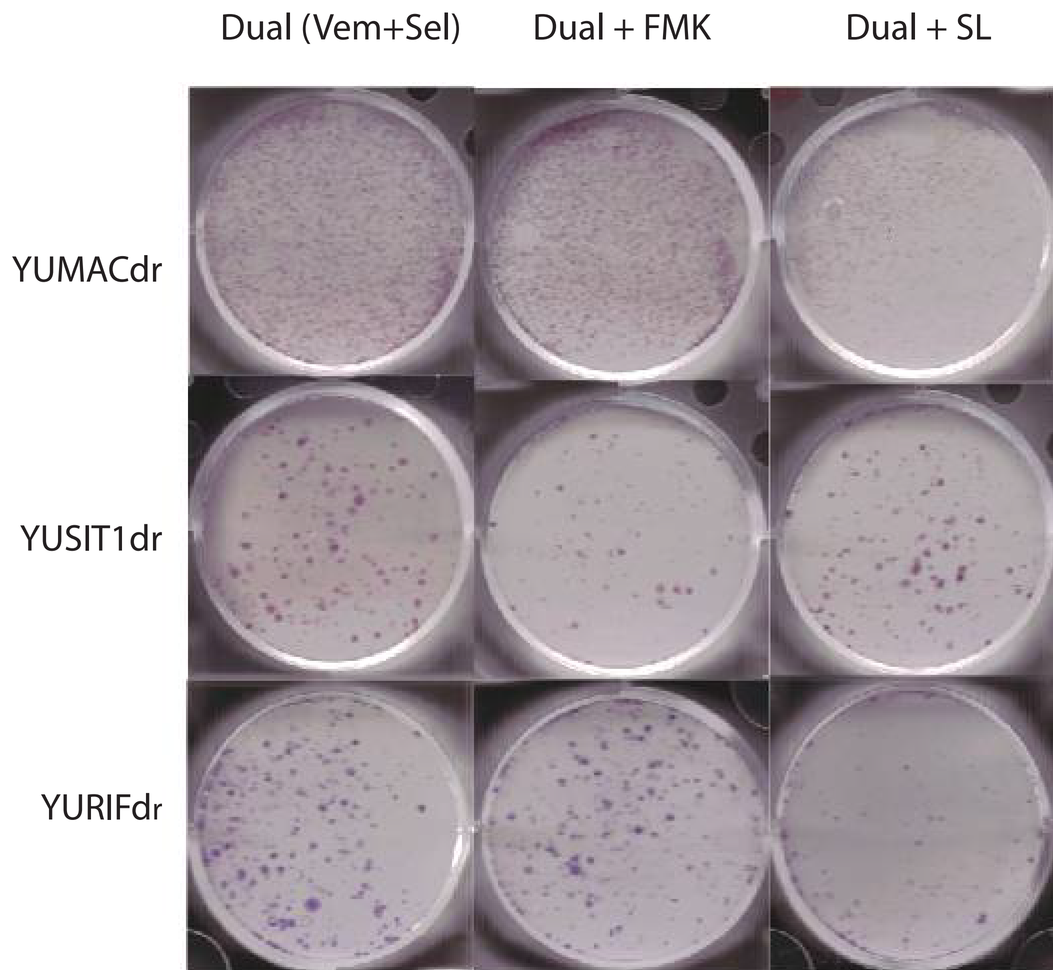



Figure S6: Long-term growth of dual resistant melanoma is moderately reduced by less-potent RSK inhibitors. 2D colony formation assays were performed with initial seeding of 5,000 cells per well. Cells were allowed to settle for 48 hours before being treated with DMSO vehicle, 10μM FMK, or 20μM SL0101 +/- 5μM vemurafenib and 150nM selumetinib for 14 days. Media and drug were replaced every 72 hours after commencement of drug treatment, N=3

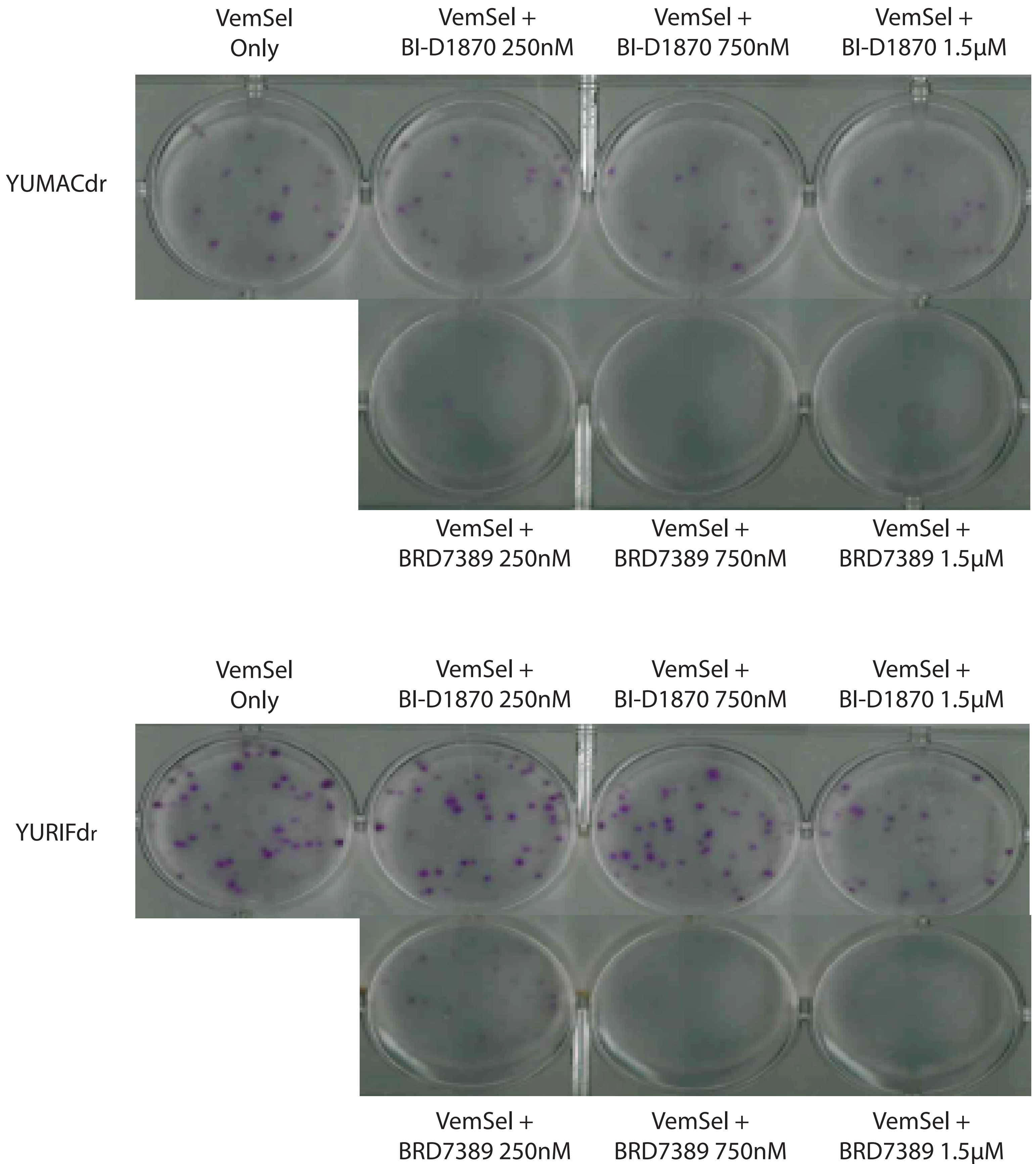



Figure S7: Reduction in colony formation occurs in a dose-dependent fashion. 2D colony formation assays were performed with initial seeding of 5,000 cells per well. Cells were allowed to settle for 48 hours before being treated with the labeled concentrations of BI-D1870 or BRD7389 and 5μM vemurafenib + 150nM selumetinib for 14 days. Media and drug were replaced every 72 hours after commencement of drug treatment, N=3

YUMACdr

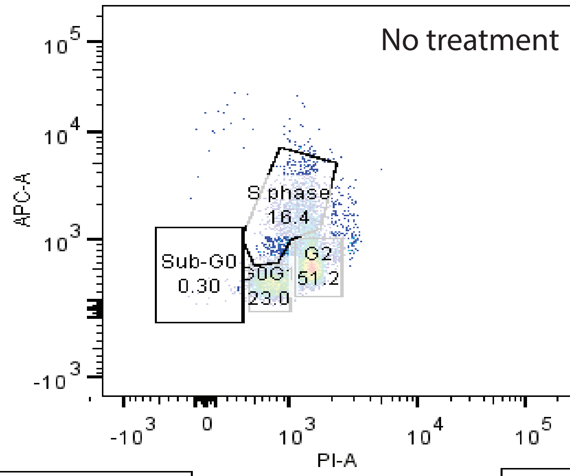
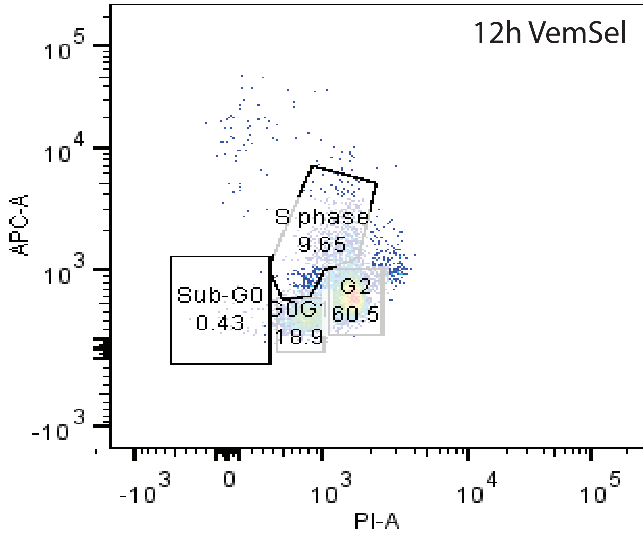
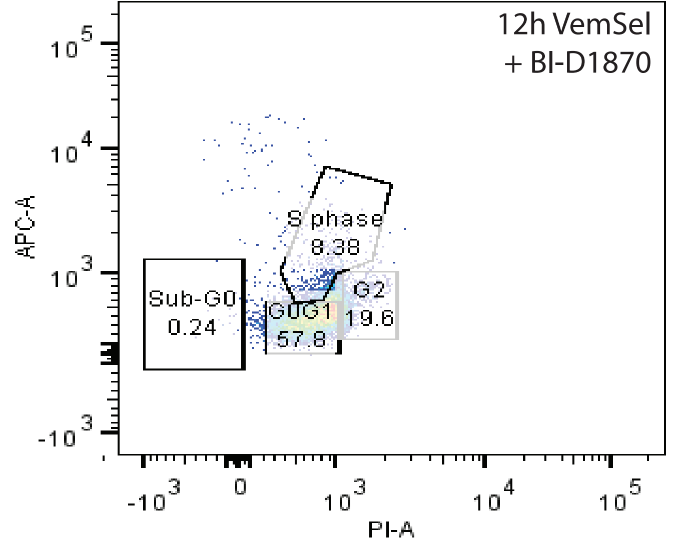
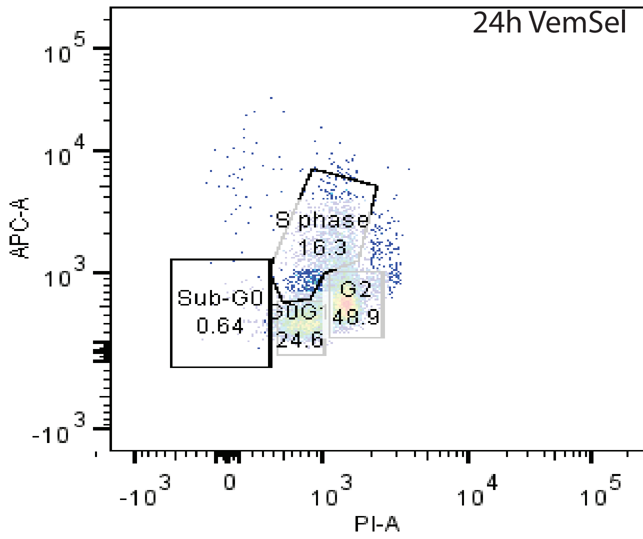
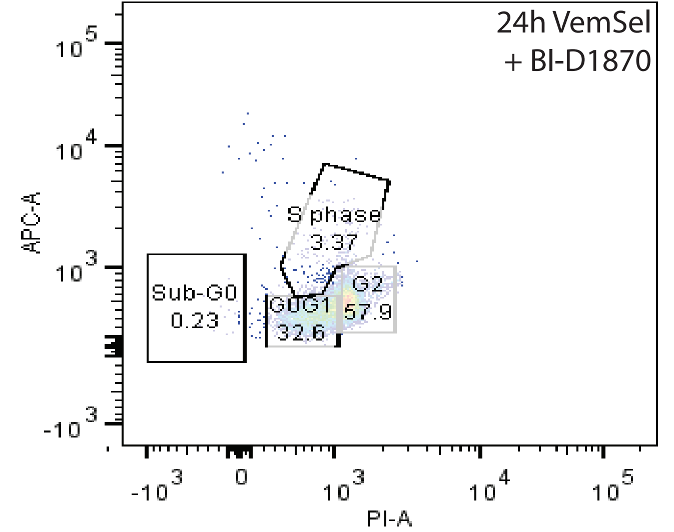


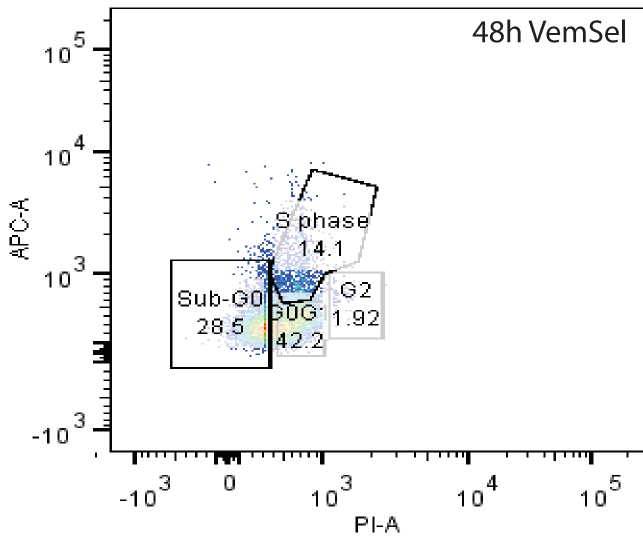

Figure S8: Combination treatment with 10µM BI-D1870 reduces proliferation in dual resistant YUMACdr more than 5µM vemurafenib + 150nM selumetinib treatment alone. Propidium iodide+BrdU cell cycle flow

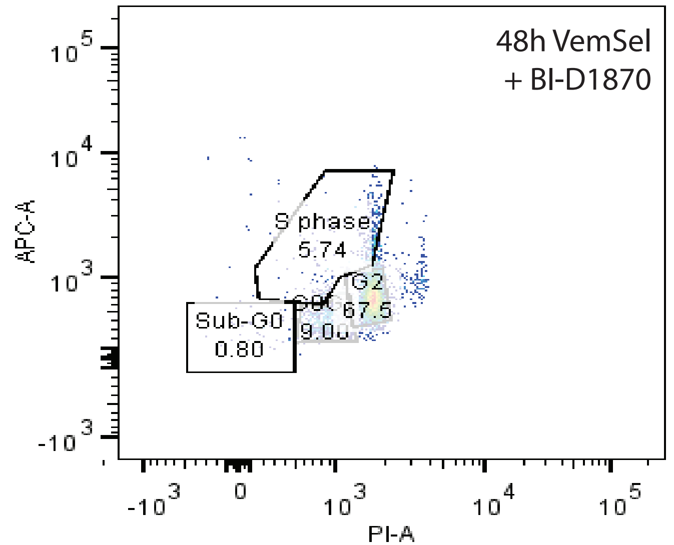


# YURIFdr

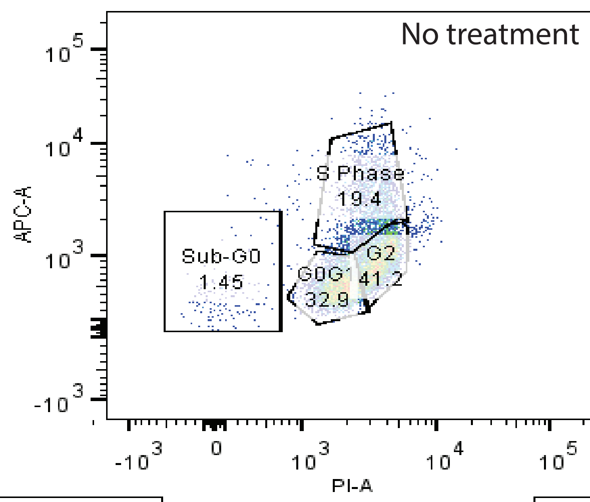
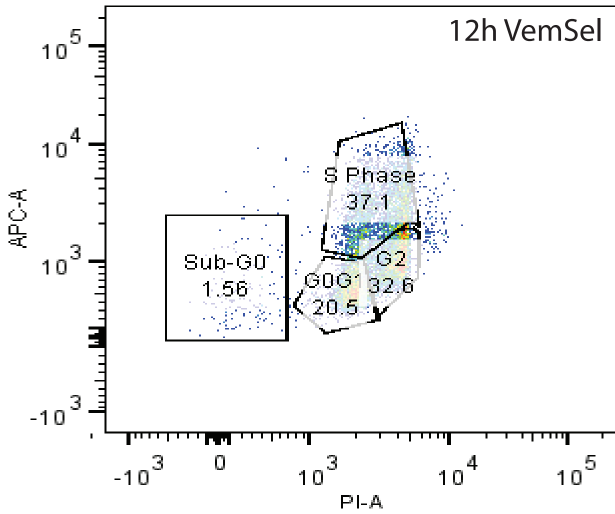
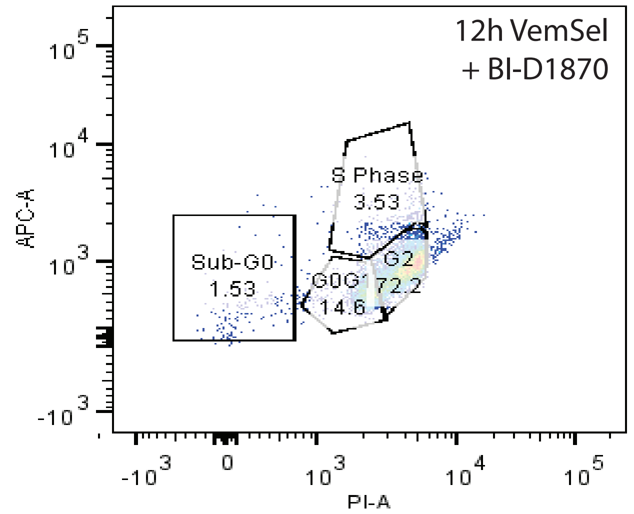
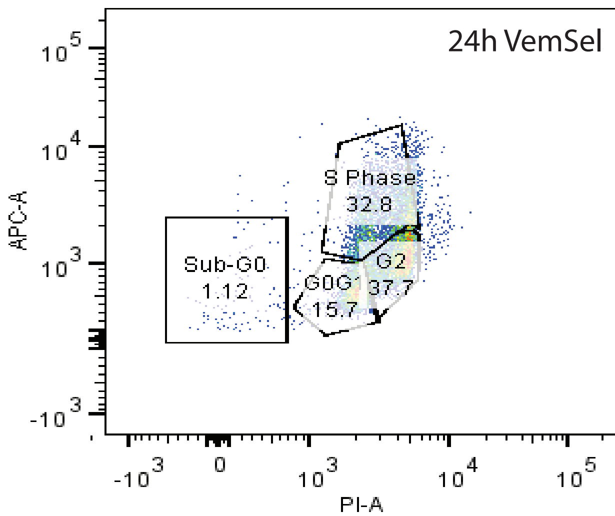
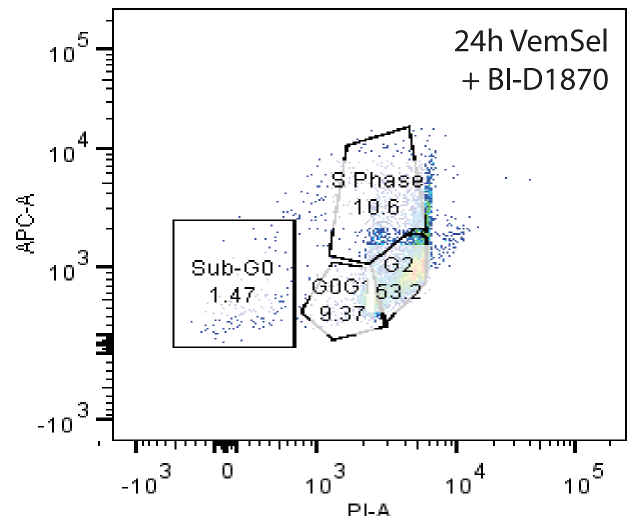


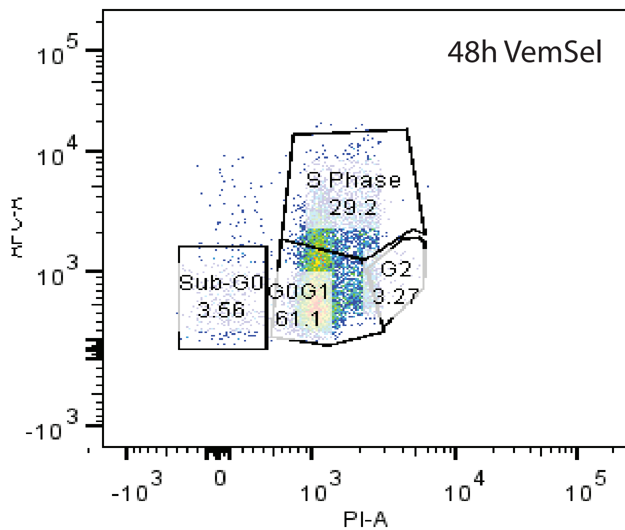

Figure S9: Combination treatment with 10μM BI-D1870 reduces proliferation in dual resistant YURIFdr more than 5μM vemurafenib + 150nM selumetinib treatment alone. Propidium iodide+BrdU cell cycle flow

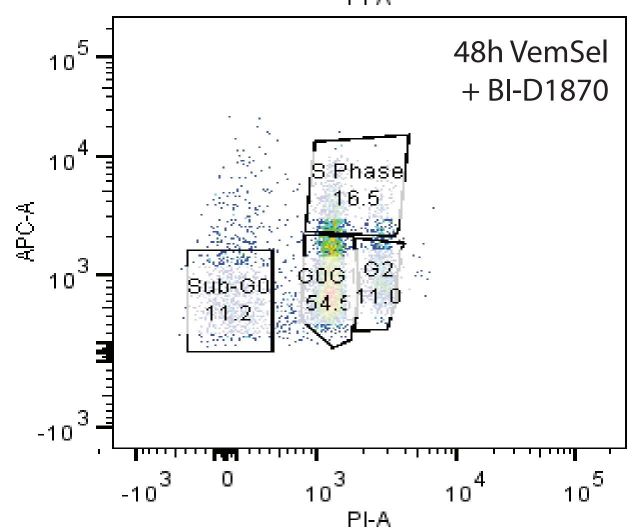


## Supplementary Materials and Methods

### Cell Culture and Generation of Acquired Resistant Lines.

Specimens were collected with patients' informed consent in accordance with the Health Insurance Portability and Accountability Act (HIPAA) under a Human Investigations Committee protocol. Expression profiling and Sanger sequencing were used to screen for mutations as described previously (Held *et al.*, 2013). All cell lines were grown in OPTI-MEM (Invitrogen, Carlsbad, CA, USA) supplemented with 1% penicillin-streptomycin and 10% fetal bovine serum maintained in a 37°C incubator maintained at 5% $CO_2$. Surgical melanoma resections were used to generate the lines and were provided by Dr. Ruth Halaban (Yale University, New Haven, CT, USA)(Halaban *et al.*, 2010). Parental YULAC, YUSIT1, and YURIF lines were continuously grown in vemurafenib (up to 5 μM) and AZD-6244 (up to 150nM) until they exhibited resistance to growth inhibition and were designated YULACdr and YUSIT1dr, and YURIFdr. Parental lines were re-designated YULACs, YUSIT1s, and YURIFs. Acquired resistance was verified over a 72 hour time period and quantified using the CyQUANT® NF Cell Proliferation Assay Kit (Life Technologies, Carlsbad, CA, USA).

### Immunoblotting

Immunoblots were conducted with the following primary antibodies all used at 1:1000: Beta Actin (cat. no. 4970; Cell Signaling, Danvers, MA, USA), Cyclin D1 (cat. no. 2978; Cell Signaling), p-p90RSK T573 (cat. no. 9346; Cell Signaling), RSK1/RSK2/RSK3 (cat. no. 9355; Cell Signaling), p-rpS6 S240/44 (cat. no. 2215; Cell Signaling), rpS6 (cat no. 2217), p-p70S6K T389 (Cell Signaling, cat. no. 9205), p70S6K (Cell Signaling, cat. no. 2708), p-Erk1/2 T202/Y204 (Cell Signaling, cat. no. 4370), total Erk1/2 (Cell Signaling, cat. no. 4370) and the secondary antibody Anti-rabbit IgG, HRP-linked Antibody (cat. no. 7074S; Cell Signaling).

### Clonogenic Assays

For Colony Formation (2-D clonogenic) assays, cells were plated at $1 \times 10^4$ cells per well in 6-well tissue culture–treated plates in triplicate and grown for 48 hours in 3 mL basal medium. Drug treatments were carried out at 48 hours and then every 4th day for a total of 3 treatments. Each time 4mL's fresh media was added. Colonies were fixed in ice-cold 100% methanol for 15 minutes and stained for 20 minutes with 0.05% crystal violet, followed by destaining with water. Plates were scanned with a VersaDoc Model 3000 imager (Bio-Rad, Hercules, CA, USA) and Quantity One software. Colonies were enumerated using ImageJ software version 1.46r.

### Flow Cytometry

For apoptosis measurement, supernatant media was collected after 72 hours. Adherent cell were trypsinized and added to the supernatant. After a single wash, pellets were stained using the BD Pharmingen Apoptosis Detection Kit II according to the manufacturer's protocol (BD Biosciences, Franklin Lakes, NJ, USA). For cell cycle analysis, cells were pulsed for 4 hours with 5-brdu (R&D Systems, Minneapolis, MN, USA) and fixed in 70% EtOH. Cells were then permeabilized with 2N HCl, which was neutralized with 0.1M borax pH 8.5. They were

subsequently stained with propidium iodide and APC-conjugated anti-brdu antibody (eBioscience, San Diego, CA, USA). Glucose uptake was assayed after cell incubation in PBS supplemented with 300 μM 2-deoxy-2-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]-D-glucose (2-NBDG) (Cayman Chemical, Ann Arbor, MI, USA) for 10 minutes. Samples were analyzed with the BD LSRII flow cytometer to at least 10,000 events per sample. Compensation for spectral overlap was applied for each experiment. Each line was treated independently, and gates were fixed based on negative control signals. Plots were generated using FlowJo 9.6.2.