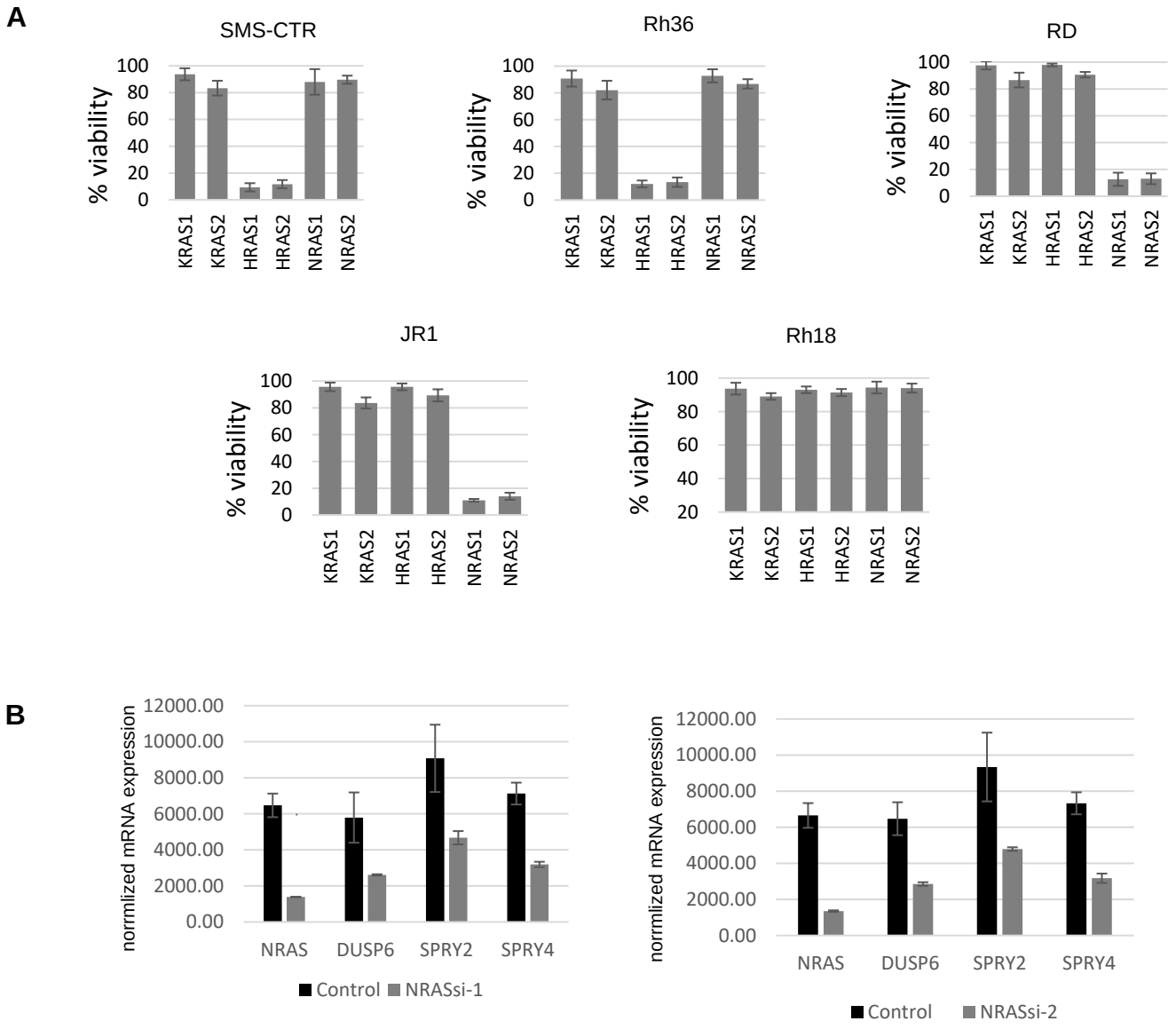
Supplementary table 1

cell line / isoform	SMS-CTR	Rh36	RD	JR1	Rh18
KRAS	WT	WT	WT	WT	WT
HRAS	Q61K	Q61K	WT	WT	WT
NRAS	WT	WT	Q61H	Q61L	WT

Supplementary table 1. Summary of RAS mutations in FN-RMS cell lines as reported in literature and confirmed with Sanger sequencing.

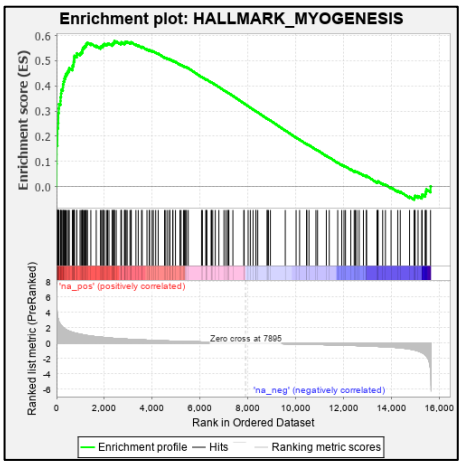
Supplementary Figure S1 (related to Figure 1)

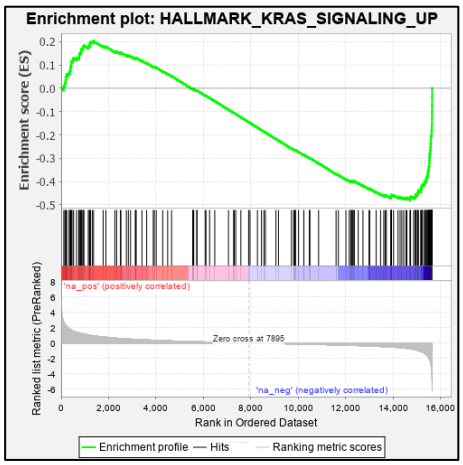


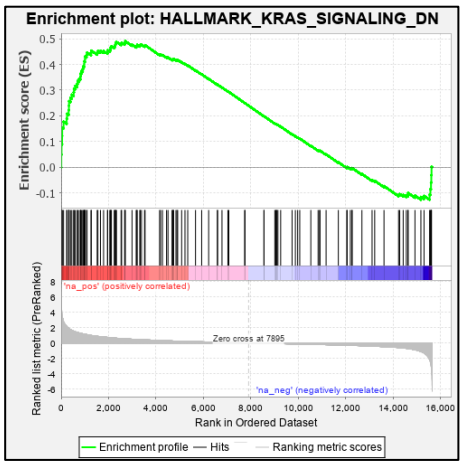
Supplementary Figure S1 continued (related to Figure 1)

C

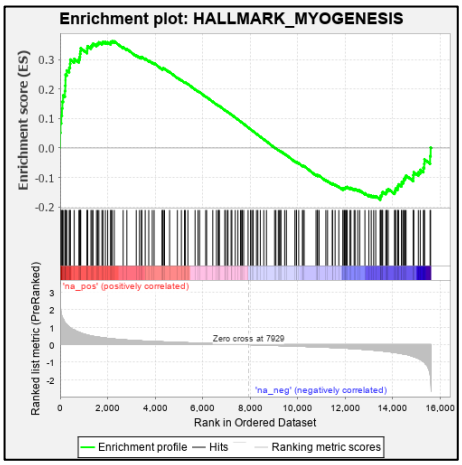
ERKi vs DMSO control

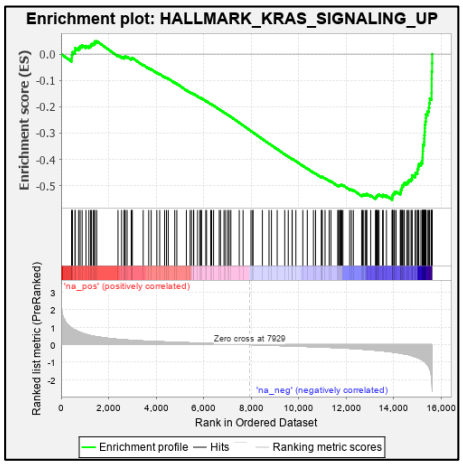


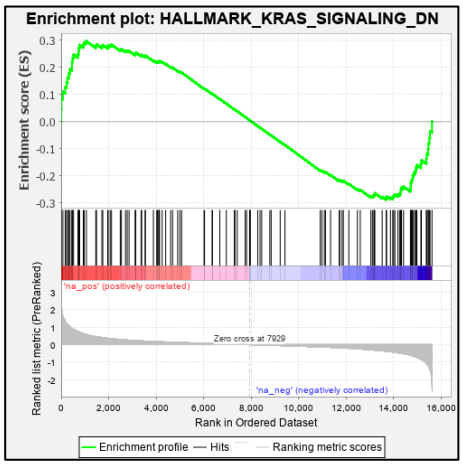




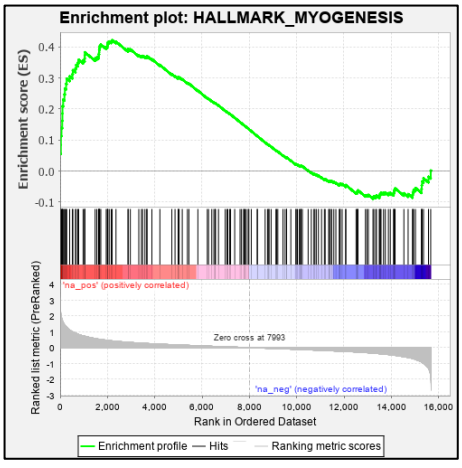
NRAS siRNA-1 vs NS control

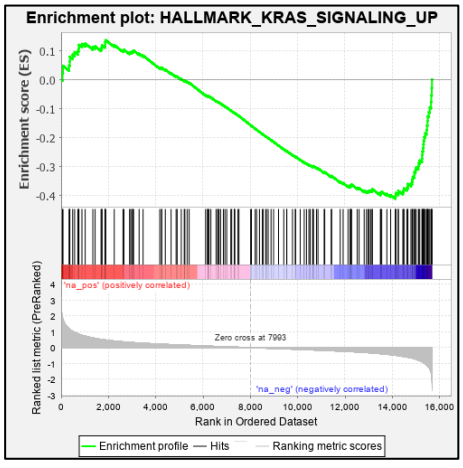


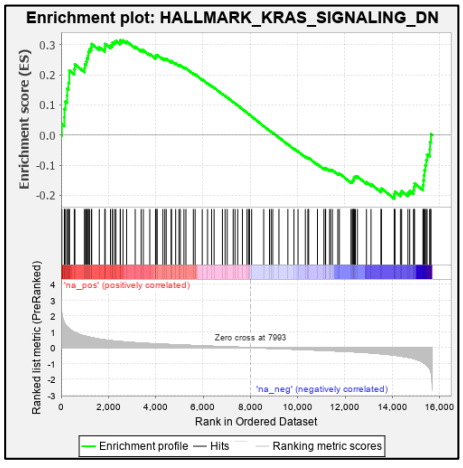




NRAS siRNA-2 vs NS control



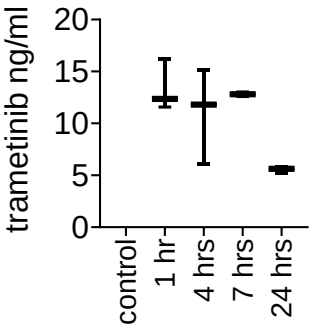




Supplementary Figure S1. ERK MAPK is essential effector of oncogenic H/NRASQ61X in FN-RMS cells. (A) Bar graphs summarizing percent viability of FN-RMS cell lines transfected with RAS isoform-specific siRNAs. Indicated cells were transfected with control or two independent siRNAs against each RAS isoform and 24 h later were plated in 96-well plates. After 8 days, viability was measured using Alamar Blue reagent. Data is presented as percent viability normalized to control siRNA transfected cells. Error bars represent SEM from three independent experiments done in triplicate. **(B)** Bar graphs summary of mRNA expression of NRAS, DUSP6, SPRY2/4 genes, following RNA sequencing of RD cells transfected with NRAS siRNAs for 48 h. Error bars represent SD from two replicate samples. **(C)** GSEA plots of hallmark gene sets for myogenesis and KRAS signaling. RD cells were treated as in figure 1D.

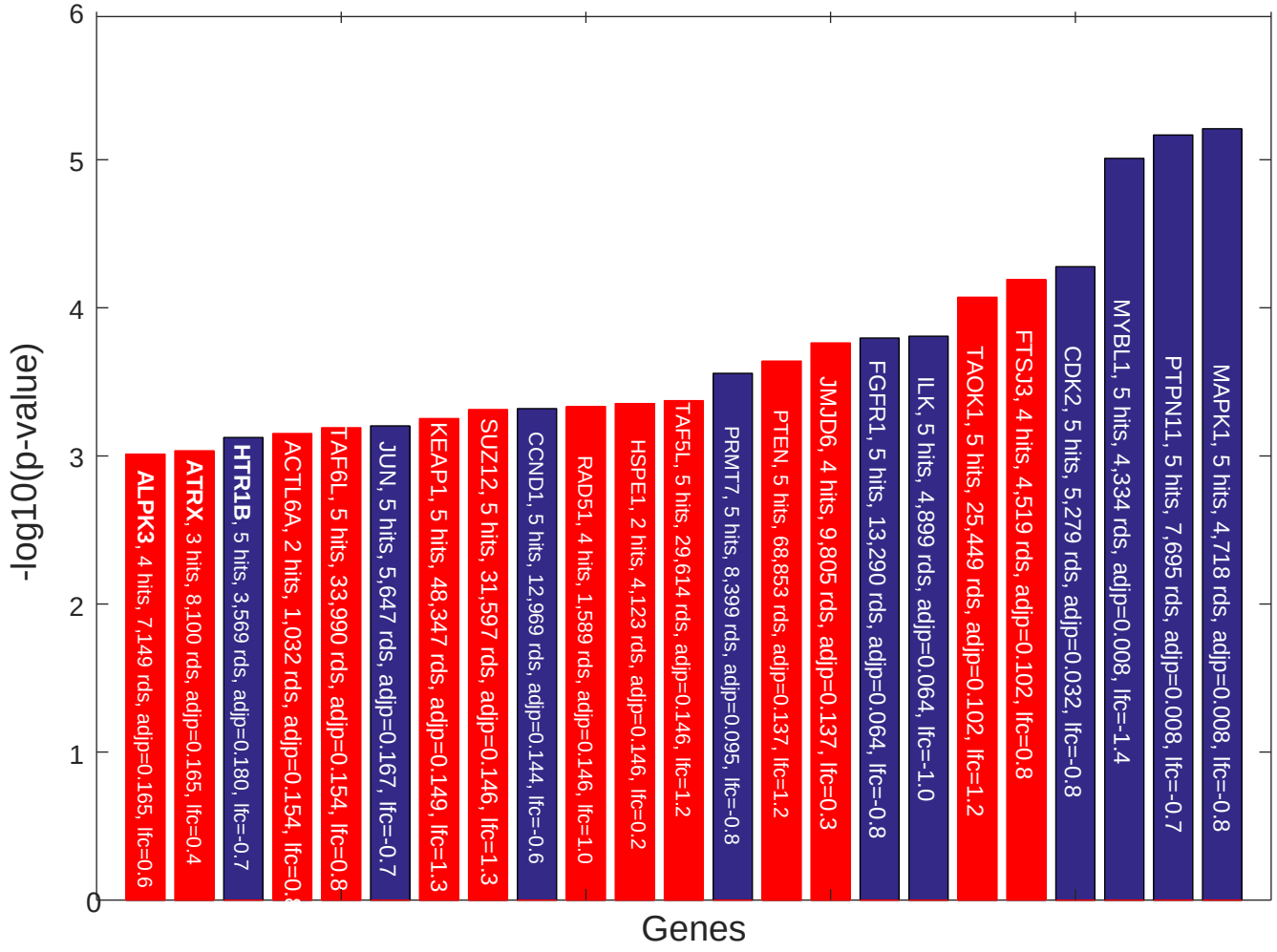
Supplementary Figure S2 (related to Figure 2)

A

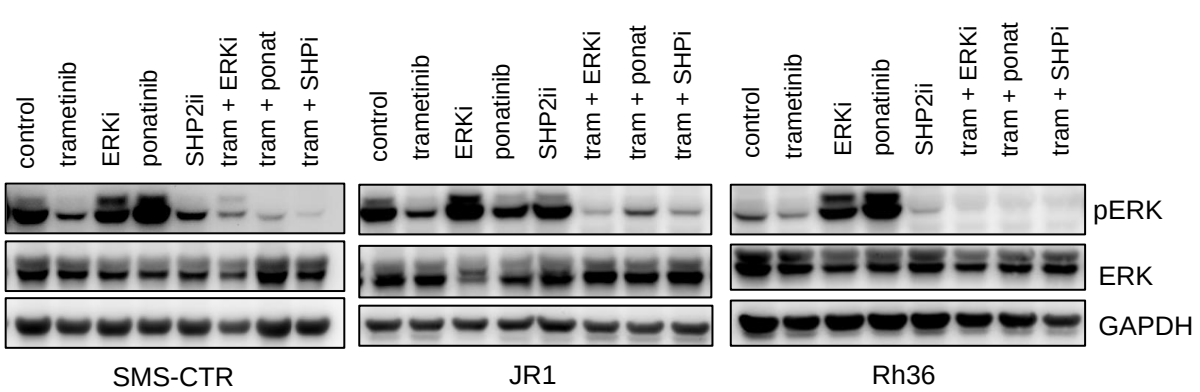


C_{max} (ng/mL)	13.37
T_{max} (hr)**	1
$AUC_{0\text{-}24hr}$ (hr*ng/mL)	235.15
$t_{1/2}$ (hr)	18.19
AUC_{inf} (hr*ng/mL)	380.93
CL/F (L/hr/kg)	2.63
V_z/F (L/kg)	68.87

B

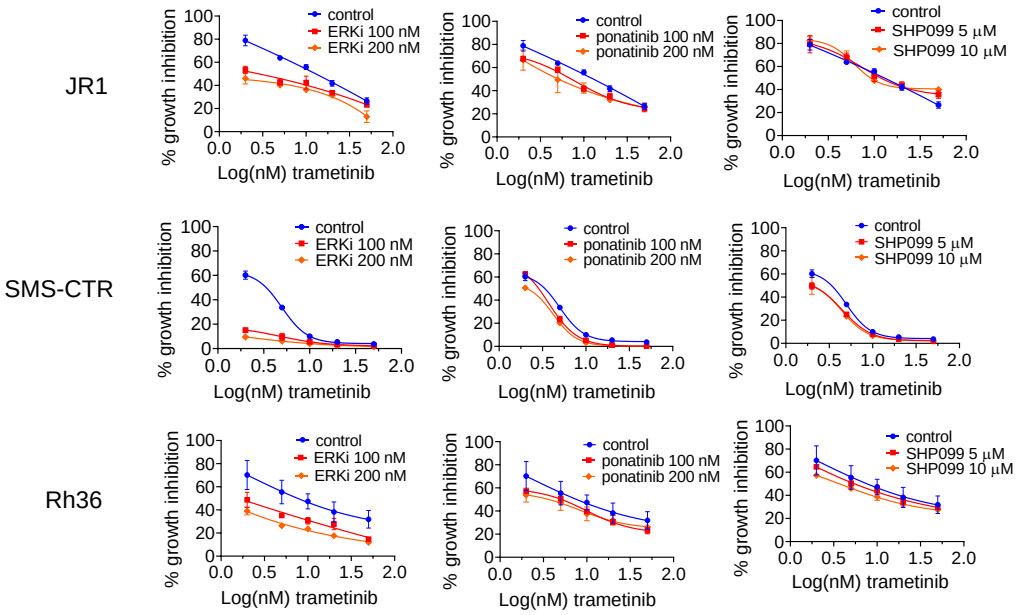


C



Supplementary Figure S2 continued (related to Figure 2)

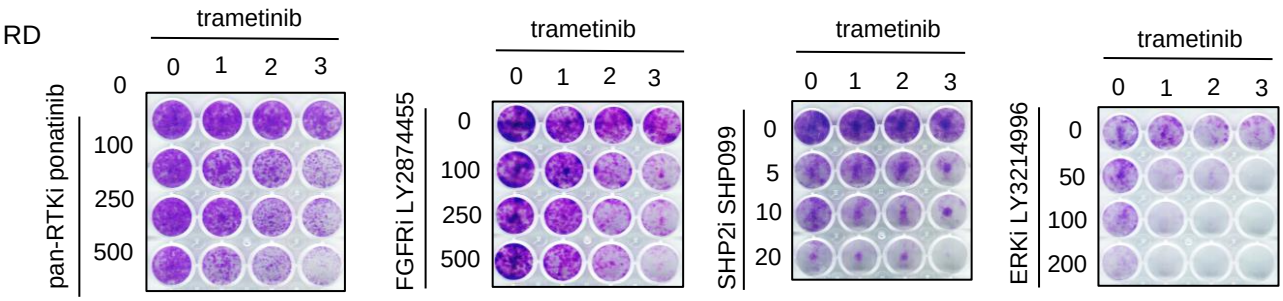
D



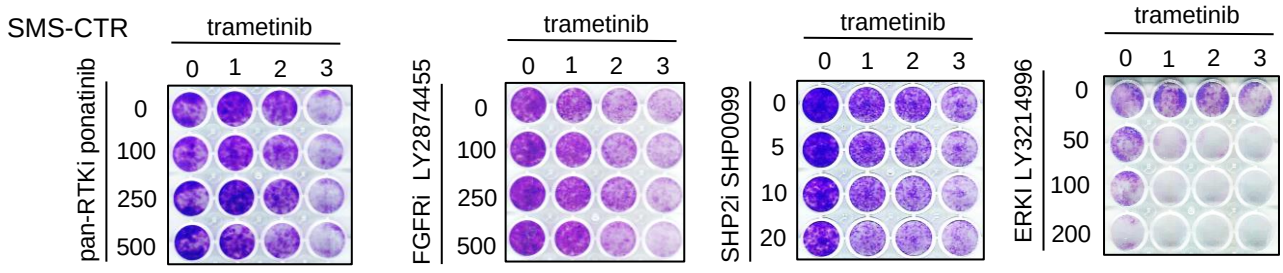
E

Cell line	CRL	ERKi		ponatinib		SHP2i	
		100 nM	200 nM	100 nM	200nM	5 uM	10 uM
JR1	12.7	2.9	<1	7.2	5.1	7.2	6.1
SMS-CTR	3.4	<1	<1	2.9	2.1	2.3	1.9
Rh36	7.6	1.6	<1	5.1	3.6	5.5	3.7

F



G



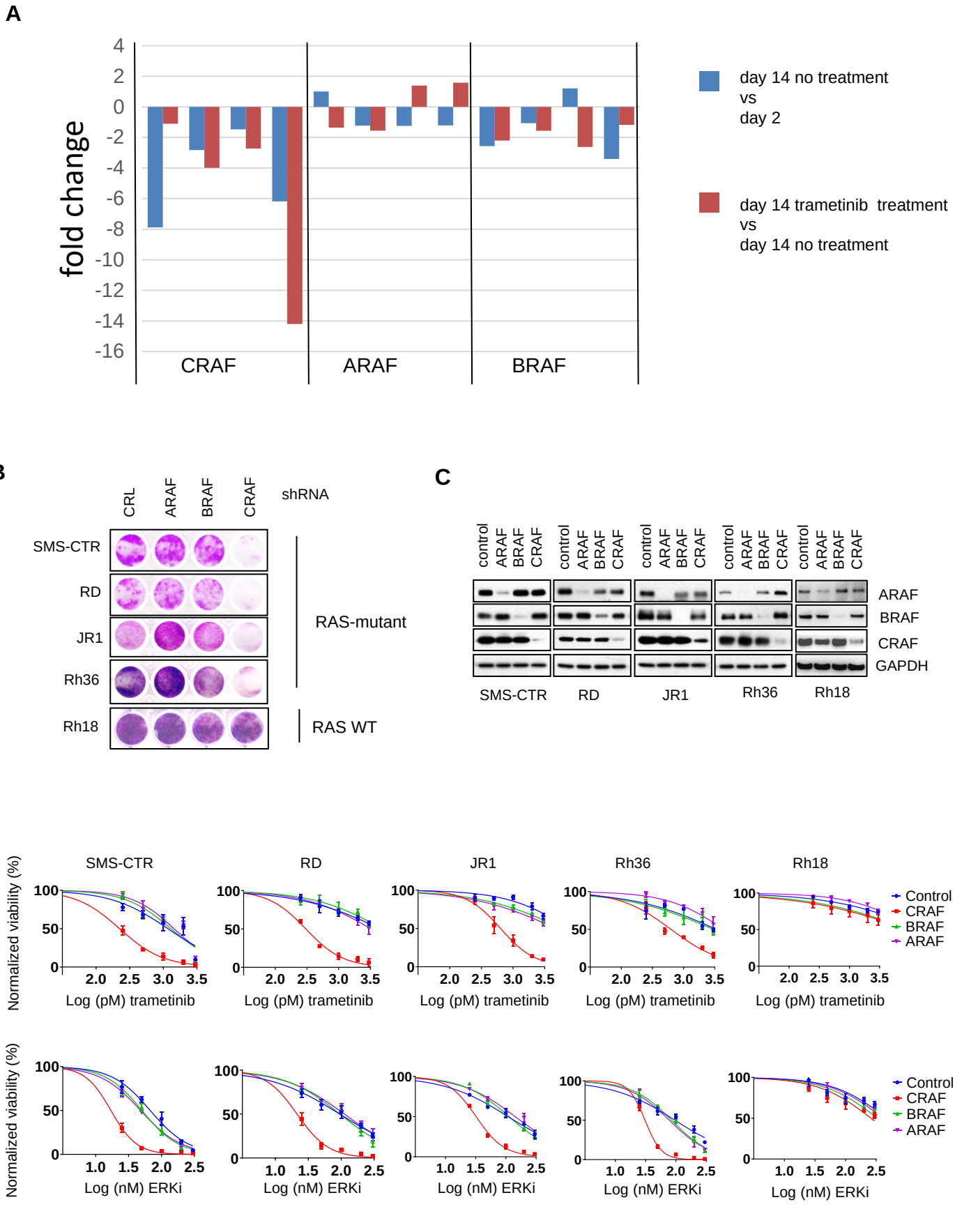
H

	ERKI LY3214996	SHP2i SHP099	FGFRi LY2874455	ponatinib
Kd or Ki (nM)	Ki= 0.65 (ERK1) Ki = 0.0063 (ERK2)	Kd= 73	NR	Kd=7.9 (FGFR1)
IC50 (uM)	0.005 (ERK1/ERK2)	0.071	0.0028	0.0022 (FGFR1)
Cmax (nM)	NR	NR	67.3	145.4

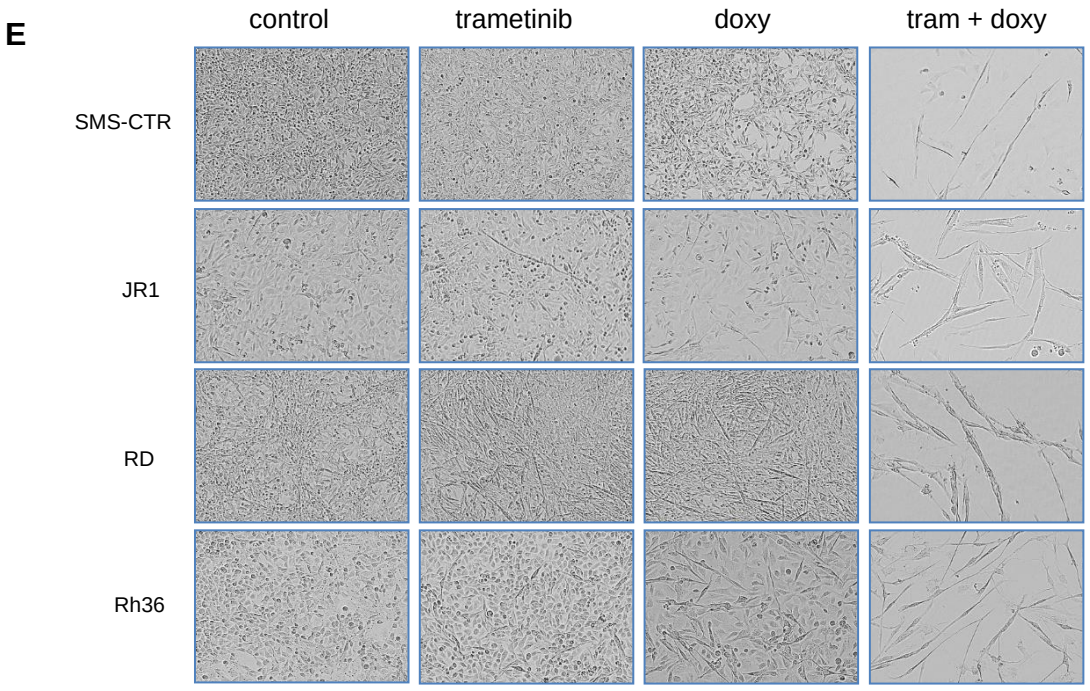
Supplementary Figure S2. **Drug-sensitizing CRISPR screen indicates vertical targeting of ERK MAPK pathway. (A)** Steady-state pharmacokinetics parameters of 1mg/kg trametinib in *C.B-Igh-1^{b}/IcrTac-Prkdc^{scid}* female mice, treated via oral gavage, every day, for 2 weeks. Left, graph showing compound plasma concentrations at indicated times. Error bars represent mean of 3 measurements ± SD. Right, table summarizing PK parameters. **(B)** Summary of top 10 positive (red) and top 10 negative (blue) hits from CRISPR library screen described in figure 2. **(C)** Western blot analysis of RAS-mutant RMS cells upon 24 h treatment with 2 nM trametinib, 50 nM ERKi LY3214996, 200 nM pan-RTKi ponatinib, 5 µM SHP2i SHP099, or indicated combinations. Phosphorylated ERK1/2 (pERK, Thr202/Tyr204), phosphorylated FRS2-α (pFRS2, Tyr436) were analyzed, total ERK1/2 and GAPDH serve as controls. **(D)** Graphs summarizing dose-response of indicated cell lines to increasing concentrations of trametinib alone (control, DMSO) and in combination with ERKi LY3214996 (left), pan-RTKi ponatinib (middle) or SHP2i SHP099 (right). Error bars represent SEM from three independent experiments done in triplicates. **(E)** Table summary of trametinib GI_{50} in (D). **(F)** Crystal violet staining images of RD cells treated with increasing concentrations of indicated inhibitors and combinations. **(G)** Crystal violet staining images of SMS-CTR cells treated as RD cells in B**. (H)** Table summary of reported biochemical properties and clinical Cmax values for the indicated inhibitors. Values are based on literature search [1-7].

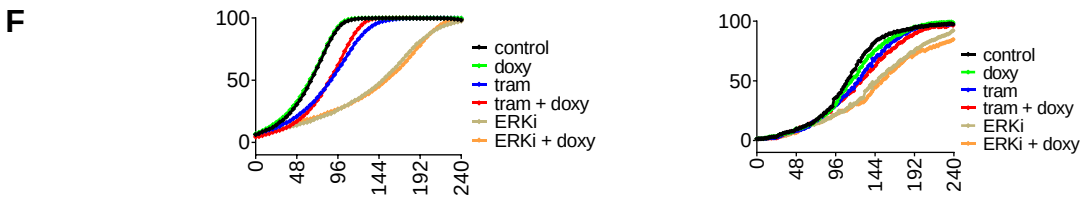
1. Chen, Y.N., et al., *Allosteric inhibition of SHP2 phosphatase inhibits cancers driven by receptor tyrosine kinases.* Nature, 2016. **535**(7610): p. 148-52.
2. Bhagwat, S.V., et al., *ERK Inhibitor LY3214996 Targets ERK Pathway-Driven Cancers: A Therapeutic Approach Toward Precision Medicine.* Mol Cancer Ther, 2020. **19**(2): p. 325-336.
3. Klein, T., et al., *Structural and dynamic insights into the energetics of activation loop rearrangement in FGFR1 kinase.* Nat Commun, 2015. **6**: p. 7877.
4. O'Hare, T., et al., *AP24534, a pan-BCR-ABL inhibitor for chronic myeloid leukemia, potently inhibits the T315I mutant and overcomes mutation-based resistance.* Cancer Cell, 2009. **16**(5): p. 401-12.
5. Cortes, J.E., et al., *Ponatinib in refractory Philadelphia chromosome-positive leukemias.* N Engl J Med, 2012. **367**(22): p. 2075-88.
6. Michael, M., et al., *A Phase 1 Study of LY2874455, an Oral Selective pan-FGFR Inhibitor, in Patients with Advanced Cancer.* Target Oncol, 2017. **12**(4): p. 463-474.
7. Zhao, G., et al., *A novel, selective inhibitor of fibroblast growth factor receptors that shows a potent broad spectrum of antitumor activity in several tumor xenograft models.* Mol Cancer Ther, 2011. **10**(11): p. 2200-10.

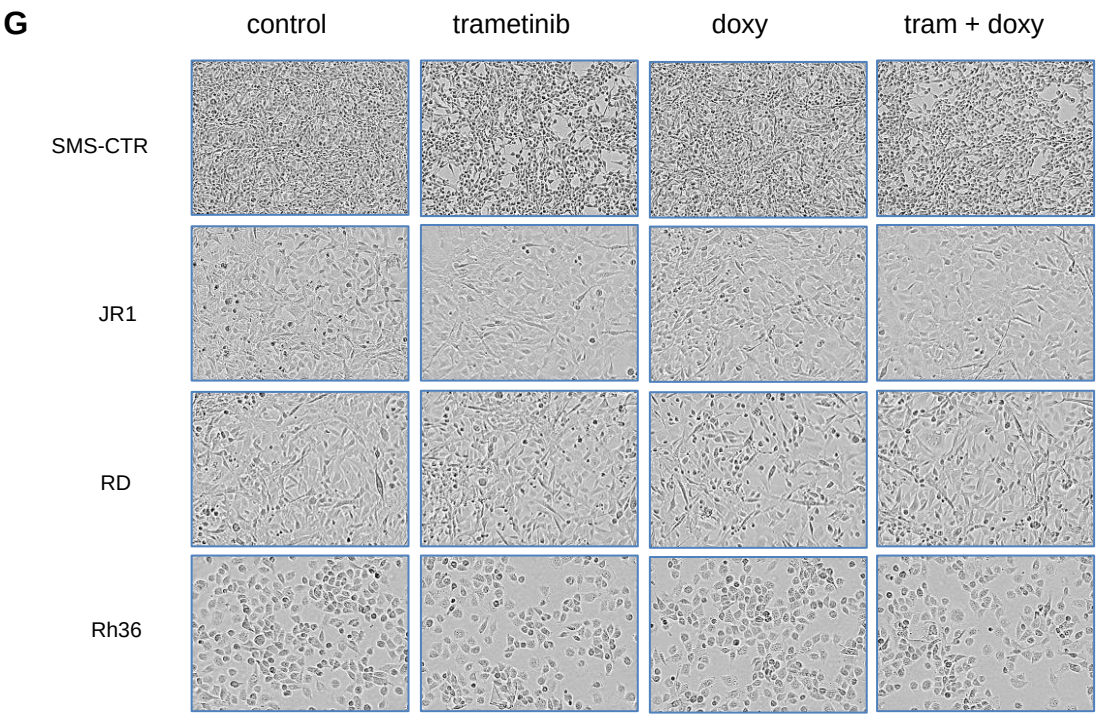
Supplemental Figure S3 (related to Figure 3)



Supplementary Figure S3 continued (related to Figure 3)

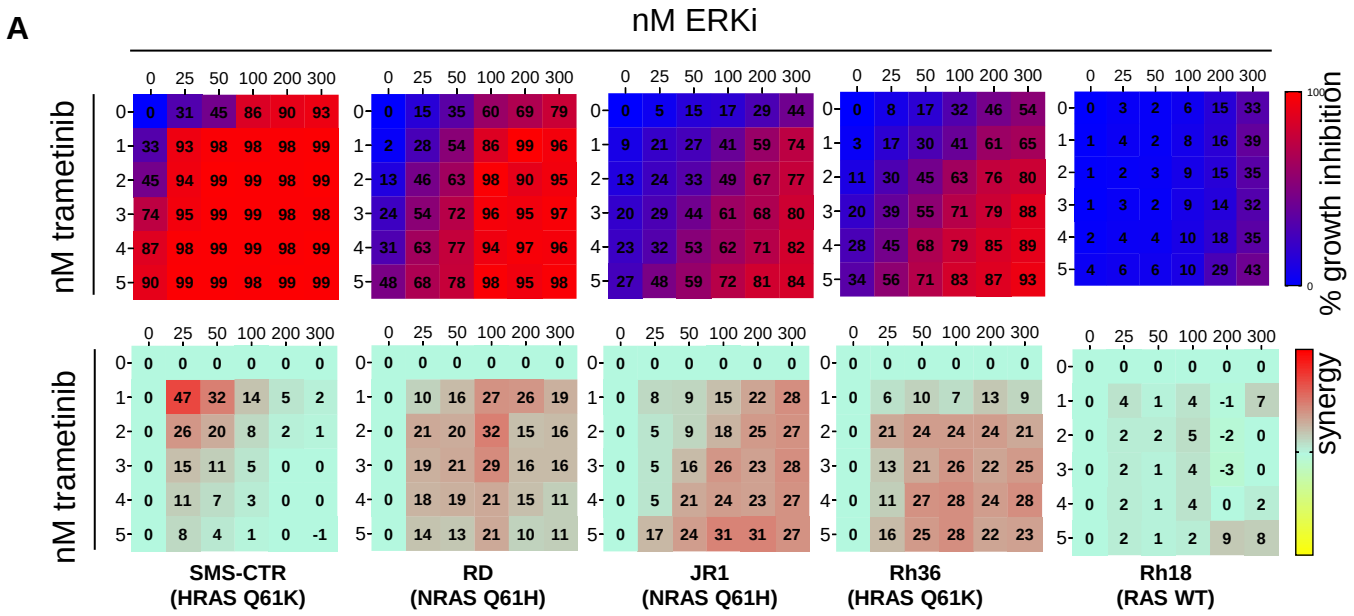


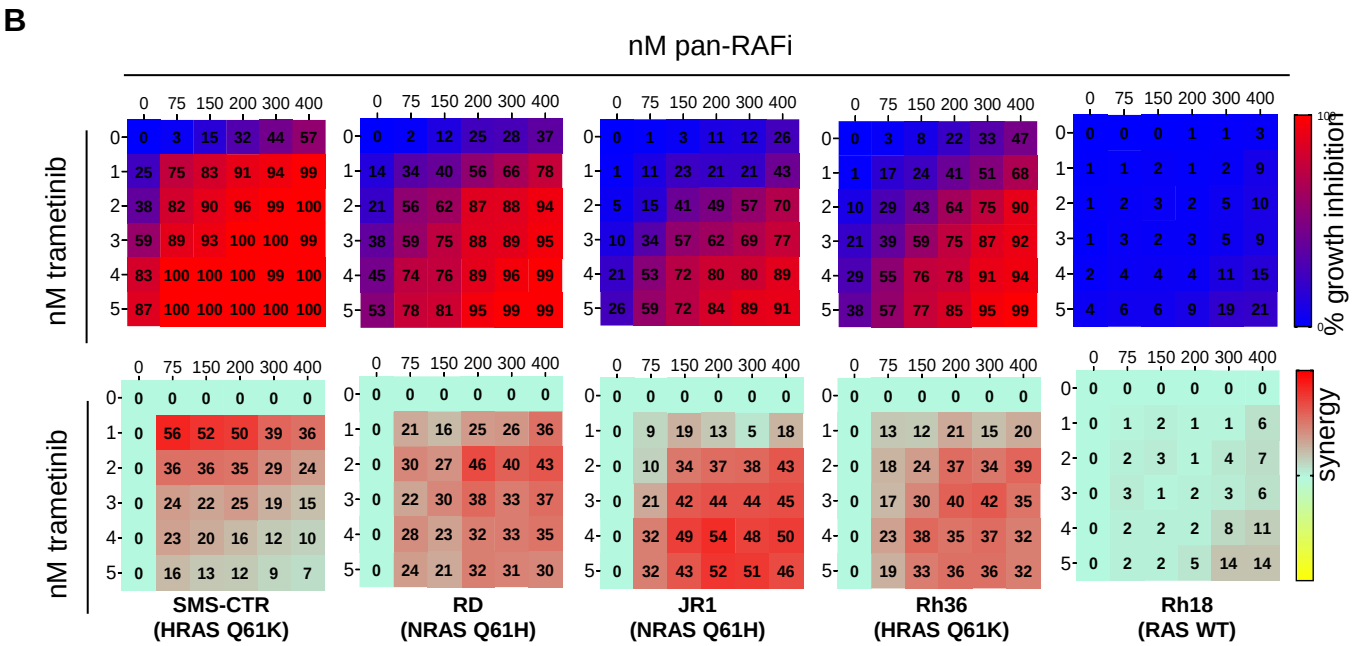




Supplementary Figure S3. Suppression of CRAF, but not A/BRAF inhibits growth and sensitize H/NRAS-mutant RMS cells to treatment with MEK or ERK inhibitors. (A) Bar graph summarizing fold change depletion of CRAF, ARAF and BRAF sgRNAs in cells infected with druggable genome CRISPR library. Shown are fold sgRNA depletion in day 14 infected cells versus day 2 infected cells (blue) and fold depletion in day 14 infected cells treated with trametinib versus day 14 infected cells without treatment (red). **(B)** Crystal violet staining images of H/NRAS-mutant RMS cells upon RAF isoforms knockdown. Indicated cells were transduced with PLKO.1 lentiviral vectors expressing shRNA against GFP (CRL), A/B/CRAF, next day selected with puromycin for 24 h, then plated at low density in 12-well plates. After two weeks, colony growth was visualized with crystal violet staining. **(C)** Western blotting images of H/NRAS-mutant RMS cells upon RAF isoforms knockdown. Indicated cells were transduced with PLKO.1 lentiviral vectors as in B, next day selected with puromycin for 24 h, then plated for crystal violet staining shown in B. Remaining cells were re-plated and collected for western blot analysis in 2 days. **(D)** Trametinib and ERKi LY3214996 dose response curves of ERMS cells upon RAF isoforms knockdown. Indicated cells were transduced with PLKO.1 lentiviral vectors and selected with puromycin as in B, then plated in 96-well plates. Next day, the cells were treated with increasing concentrations of trametinib (top) or LY3214996 (bottom) for 96 h and viability was measured with Alamar Blue reagent. Percent normalized viability was plotted using GraphPad Prism. Error bars represent SEM from three experiments done in triplicate. **(E)** Bright field images of ERMS cell lines expressing dox-inducible shRNA against CRAF. Images were taken with Incucyte at the 240 h timepoint in the experiment described in figure 3E. **(F)** Growth curves of FN-RMS cells expressing doxycycline-inducible non-specific (control) shRNA. SMS-CTR or RD cells were transduced with pSMART lentiviral vector expressing doxycycline-inducible control shRNA, selected with puromycin for 2 days and plated in 96 well plates. Next day, the cells were treated with DMSO, 200 ng/ml doxycycline, 2 nM trametinib +/- doxycycline or 100 nM LY3214996 +/- doxycycline. Percent confluency was determined by imaging with Incucyte every 2 h for 240 h. **(G)** Bright field images of ERMS cell lines expressing doxycycline-inducible control shRNA. Images were taken with Incucyte at the 240 h timepoint in the experiment described in figure S3F.

Supplementary Figure S4 (related to Figure 4)

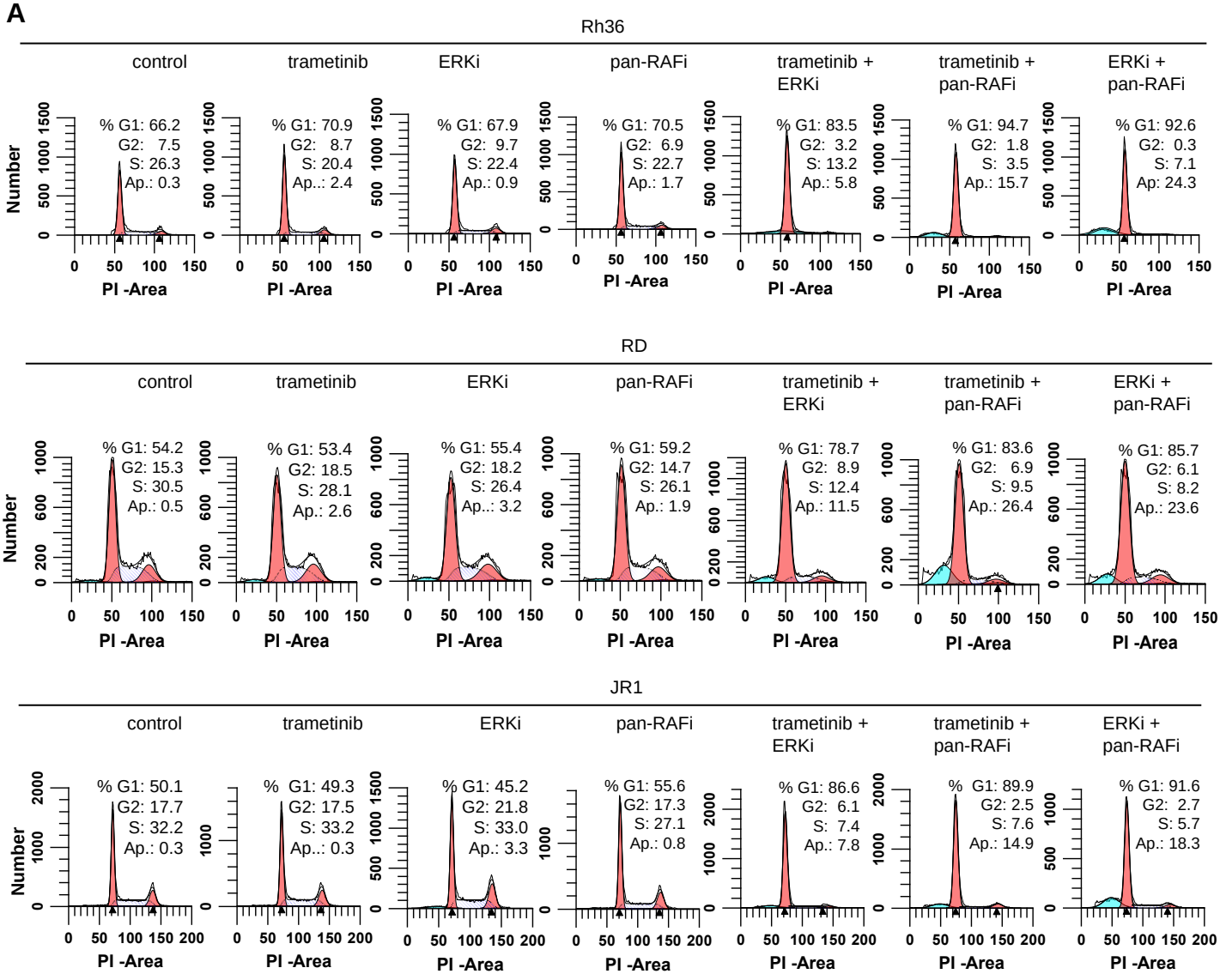




Supplementary Figure S4. Vertical targeting of MAPK pathway synergistically inhibits H/NRAS-mutant RMS cell growth. Cells were plated in 96 well plates and treated with increasing concentrations of trametinib, ERKi LY3214996 or combinations **(A)**, or increasing concentrations of trametinib, pan-RAFi LY300912 or combinations **(B)** for 5 days. Viability was measured with Alamar Blue reagent and Bliss scores were calculated using Combenefit.

Supplementary Figure S5 (related to Figure 5)

A



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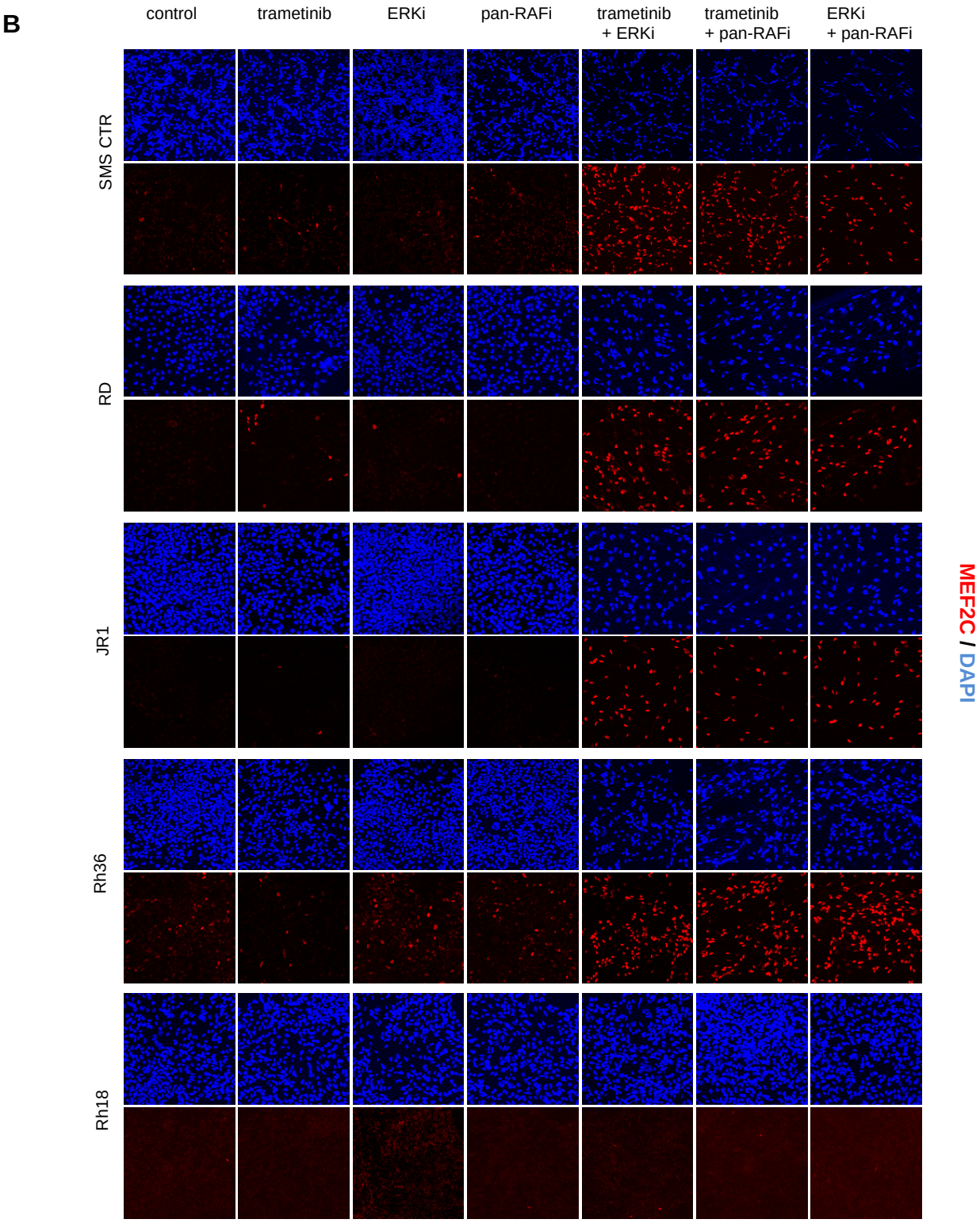
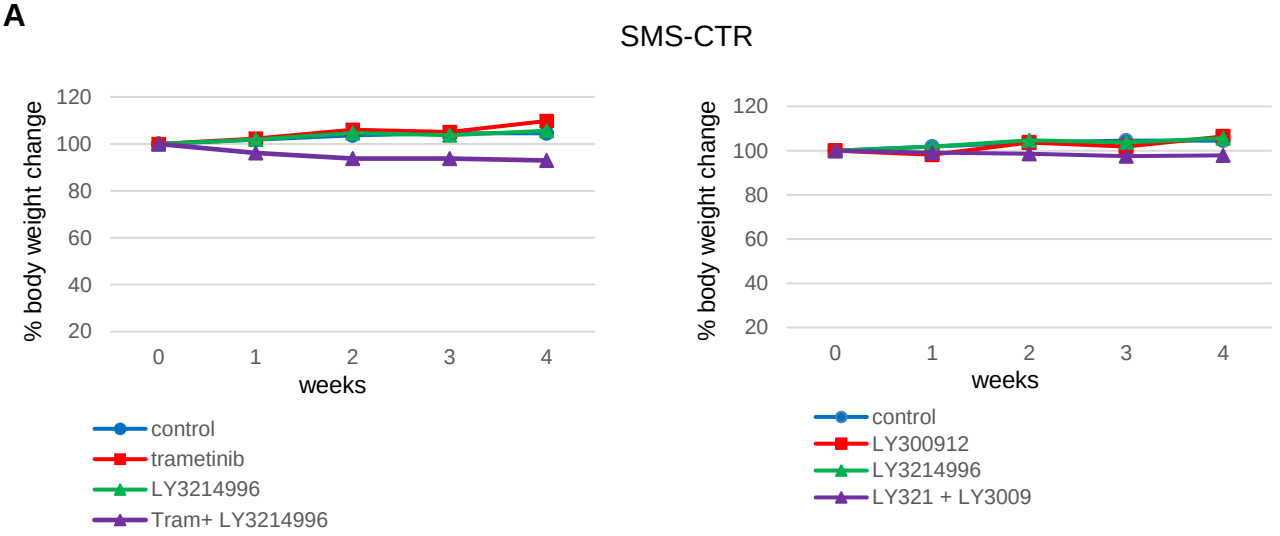


Supplementary Figure S5. Vertical targeting of MAPK pathway induces G1 cell cycle arrest, apoptosis and differentiation in RAS-mutant, but not RAS-wild-type ERMS cells. (A) Cell cycle distribution and sub-G1 quantification in ERMS cells upon treatment with 2 nM trametinib, 100 nM ERKi LY3214996, 100nM pan-RAFi LY300912 or combinations for 72 h, followed by propidium iodide staining, flow cytometry and analysis with Modfit software. **(B)** Immunofluorescence staining images for MEF2C of indicated cell lines following treatment with 2 nM trametinib, 100 nM LY3214996, 100nM LY300912 or combinations for 8 days. Scale bars, 200 µm.

Supplementary Figure S6 (related to Figure 6)

A



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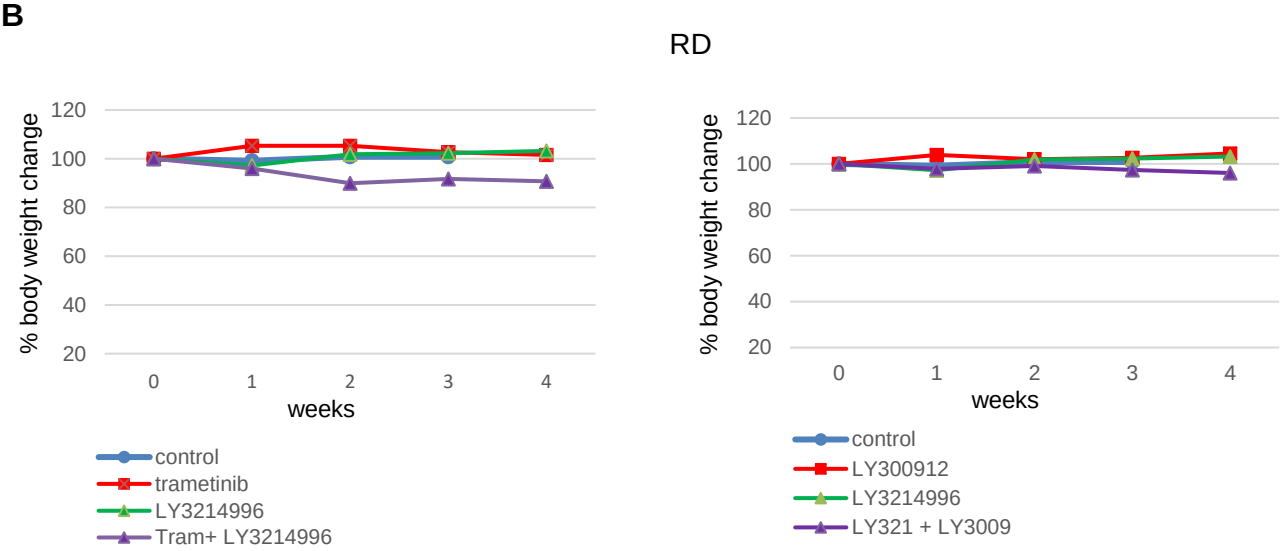

Supplementary figure S6. Graphs summarizing percent body weight change of mice bearing SMS-CTR **(A)** and RD **(B)** tumor xenografts treated as in figures 6A and 6B respectively.