Supplementary Information

Title: RAS-MAPK dependence underlies a rational polytherapy strategy in EML4-ALK positive lung cancer

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Supplemental Figures and Legends.

Figure S1

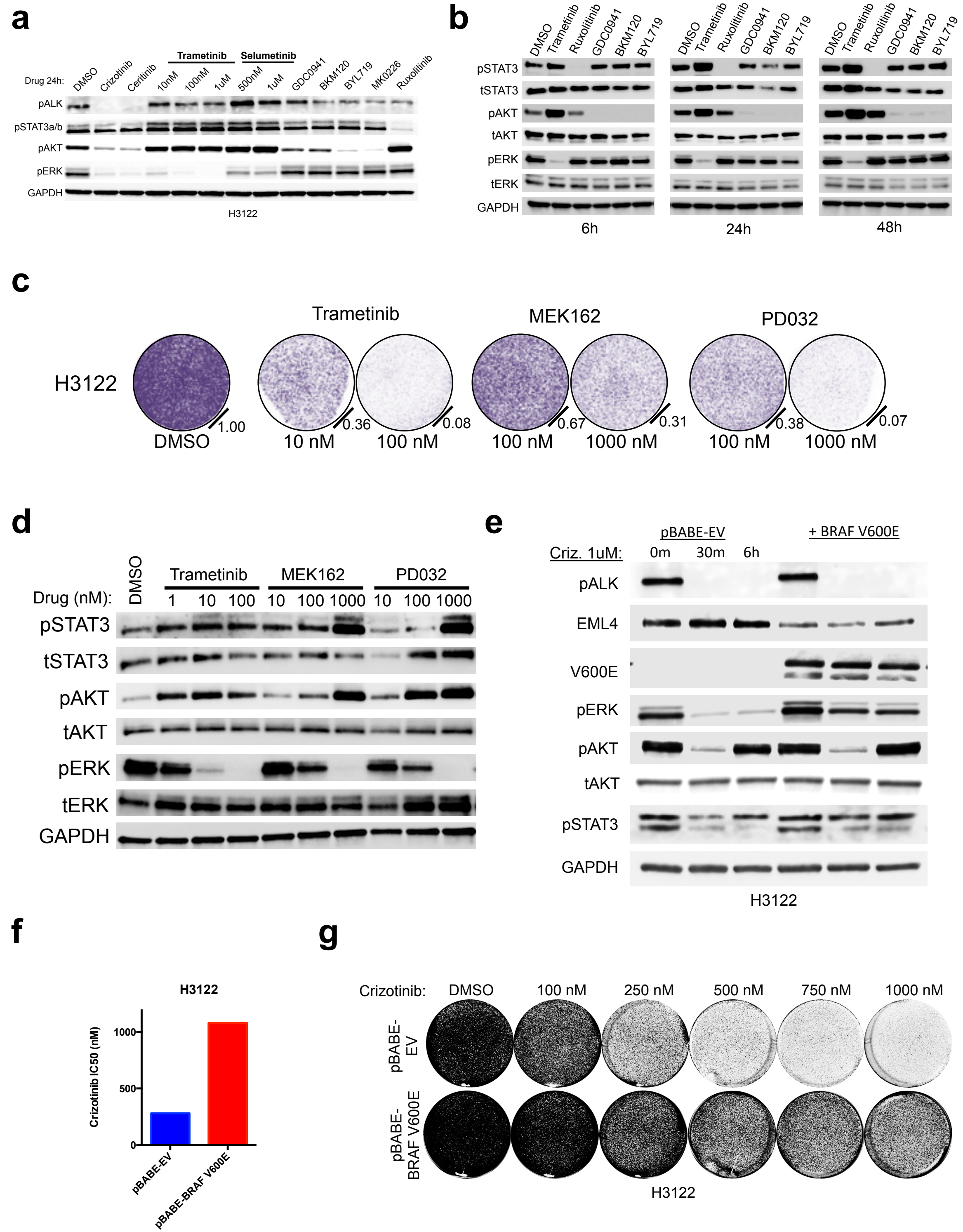

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Figure S1. RAS-MAPK signaling regulates oncogene dependence in EML4-ALK lung adenocarcinoma cells. (**a**) Immunoblot analysis using indicated antibodies in H3122 cells treated with inhibitors (24h) from **Fig. 1c**. Target inhibition at concentrations used is shown. **(b)** Immunoblot analysis using the indicated antibodies in H3122 cells treated with indicated inhibitors at concentrations from **Fig. 1c** for 6h, 24h, or 48h, indicating duration of target inhibition up to 48h (at the time of media change for the growth assay in **Fig. 1c**). **(c-d)** H3122 cells were treated with several different MEK inhibitors (trametinib, MEK162, PD032) at the indicated concentrations and **(c)** viability was measured over a 5-day crystal violet assay. **(d)** Immunoblot analysis of H3122 cells with the indicated antibodies treated with the indicated MEK inhibitors for 24h. (**e-g**) H3122 cells were transduced with $BRAF^{V600E}$ and then (**e**) immunoblot analysis was performed on cells treated with 30m or 6h crizotinib (1uM), (**f**) IC50 values were estimated from crizotinib dose response (cell titer glo), and (**g**) cells were plated and subjected to a 5-day growth assay in the presence of indicated concentrations of drug, then fixed and stained with crystal violet. All data shown represent at least 3 independent experiments.

Figure S2

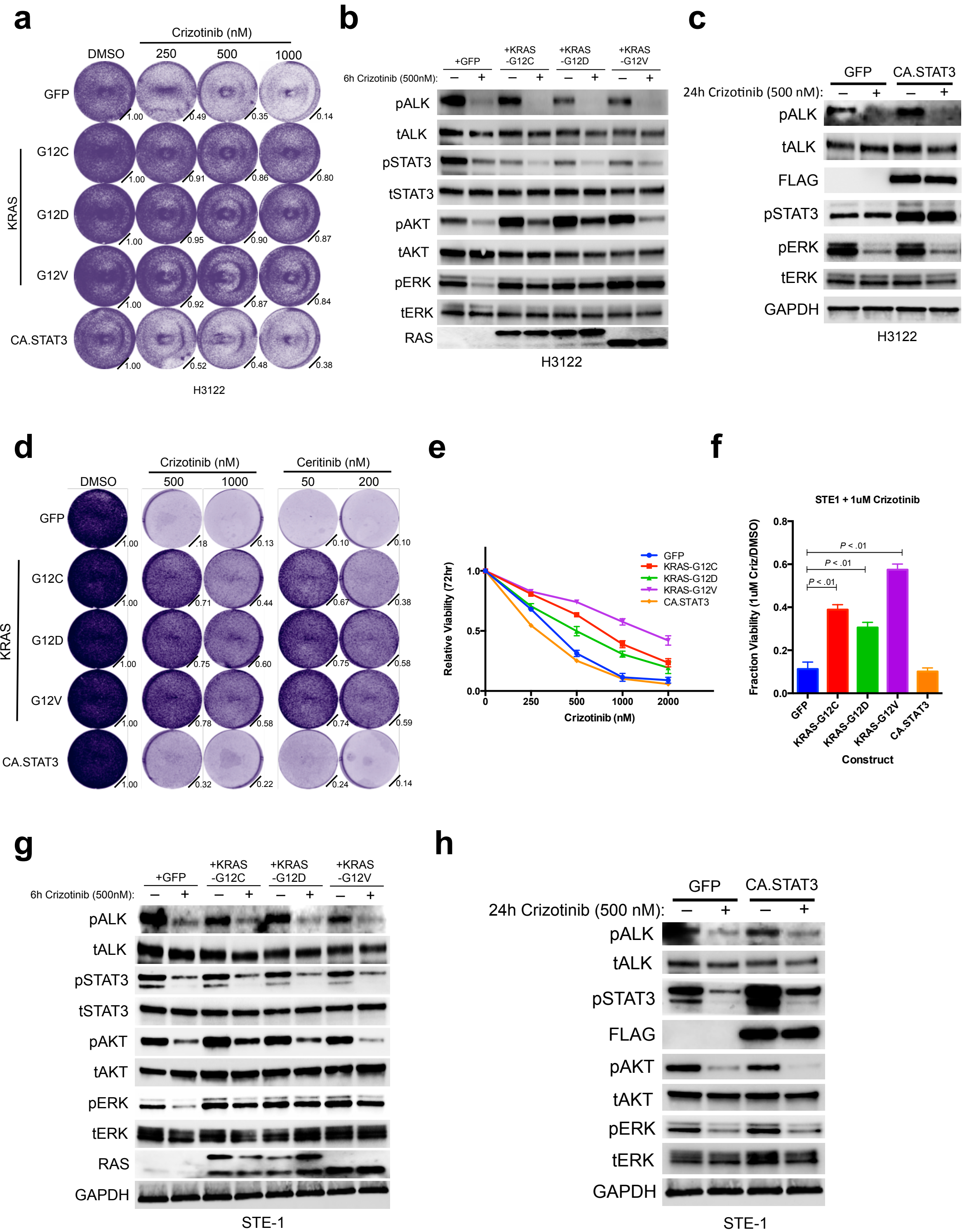

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Figure S2. Activation of RAS signaling promotes resistance to ALK inhibitors via rescue of MAPK signaling. (a-c) H3122 cells expressing the indicated cDNAs (GFP, $KRAS^{G12C/D/V)}$, or CA.STAT3) were assayed for **(a)** viability in the presence of crizotinib and **(b-c)** subjected to immunoblot analysis with the indicated antibodies and drug treatments. **(d-h)** STE-1 cells expressing the indicated cDNAs (GFP, $KRAS^{G12C/D/V)}$, or CA.STAT3) were assayed for **(d)** viability in the presence of crizotinib using either **(d)** crystal violet assay or **(e-f)** 72h Cell Titer Glo assay. These same cells were then subjected to **(g-h)** immunoblot analysis with indicated antibodies and indicated treatments. All data shown represent at least 3 independent experiments.

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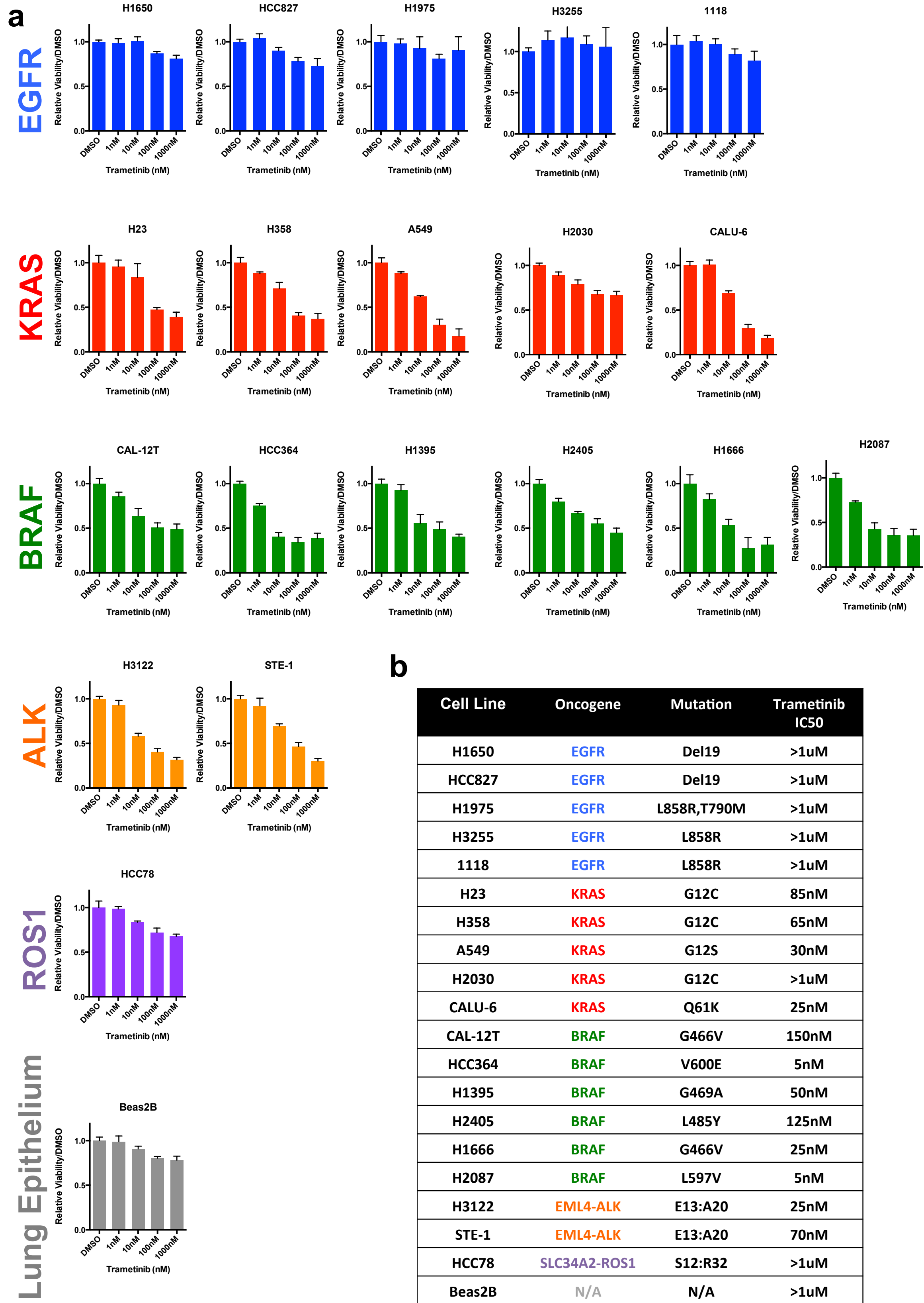
Figure S3



Cell Line	Oncogene	Mutation	Trametinib IC50
H1650	EGFR	Del19	>1uM
HCC827	EGFR	Del19	>1uM
H1975	EGFR	L858R,T790M	>1uM
H3255	EGFR	L858R	>1uM
1118	EGFR	L858R	>1uM
H23	KRAS	G12C	85nM
H358	KRAS	G12C	65nM
A549	KRAS	G12S	30nM
H2030	KRAS	G12C	>1uM
CALU-6	KRAS	Q61K	25nM
CAL-12T	BRAF	G466V	150nM
HCC364	BRAF	V600E	5nM
H1395	BRAF	G469A	50nM
H2405	BRAF	L485Y	125nM
H1666	BRAF	G466V	25nM
H2087	BRAF	L597V	5nM
H3122	EML4-ALK	E13:A20	25nM
STE-1	EML4-ALK	E13:A20	70nM
HCC78	SLC34A2-ROS1	S12:R32	>1uM
Beas2B	N/A	N/A	>1uM

Figure S3. MEK dependence in EML4-ALK and other lung adenocarcinoma cell lines. (**a**) The indicated cell lines (all patient-derived lung cancer lines, except Beas2B which are immortalized human bronchial epithelial cells lacking a driver oncogene) were treated with increasing doses of trametinib (1nM, 10nM, 100nM, and 1000nM) for 72h and viability was measured using cell titer glo. (**b**) Table showing the indicated cell lines tested and their respective genetic drivers. IC50 values were estimated from growth curves in (**a**). ($n \geq 3$, data are shown $\pm$ SEM for quantitative assays). All data shown represent at least 3 independent experiments.

Figure S4

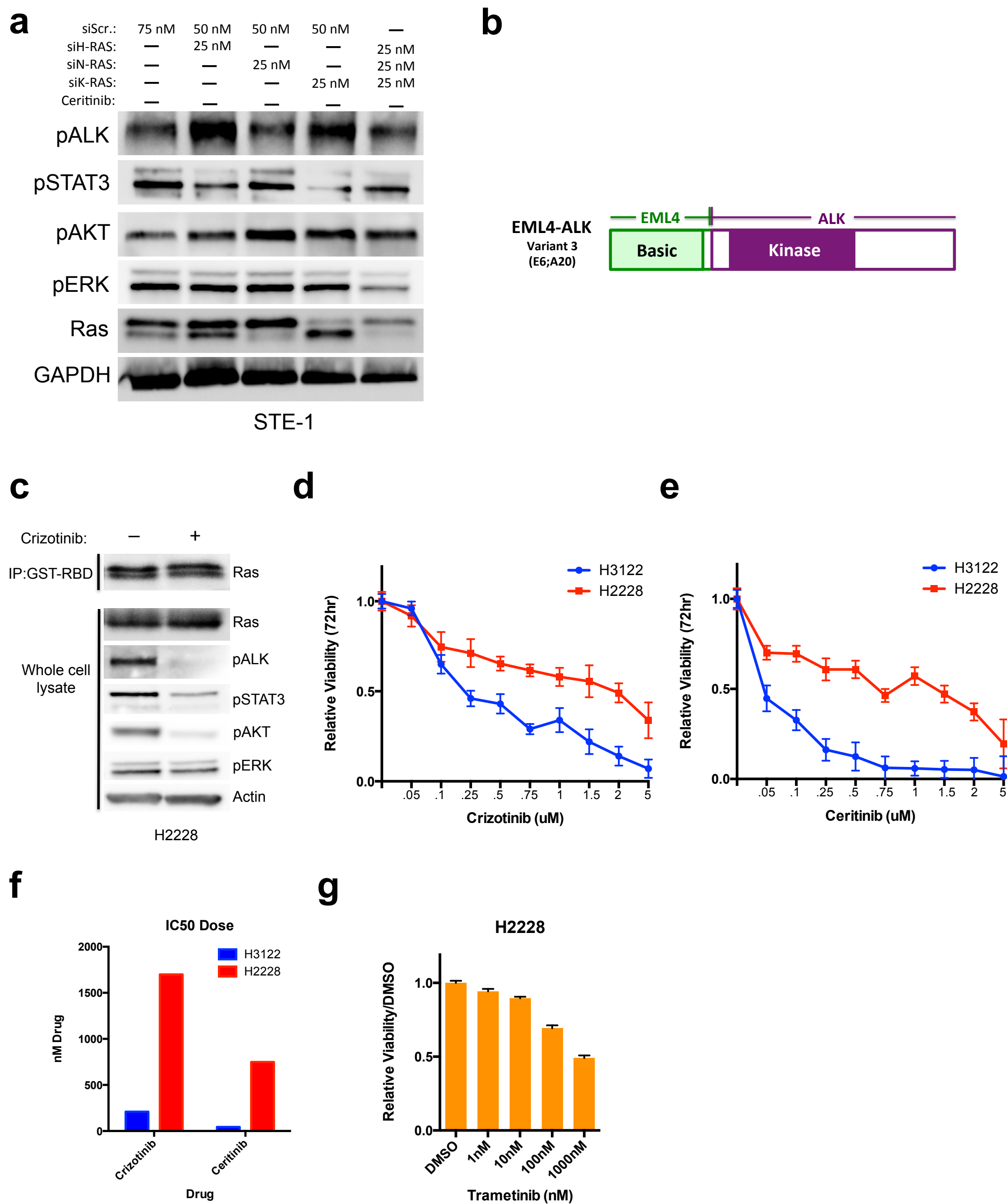


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Figure S4. EML4-ALK engages H-, N-, K-RAS via the EML4 HELP domain. (**a**) Immunoblot analysis with indicated antibodies on STE-1 cell lysates transfected with indicated siRNAs at the indicated concentrations for 72h. **(b)** Graphical depiction of EML4-ALK variant 3 present in H2228 lung adenocarcinoma cells and that lacks the HELP domain in EML4, in contrast to EML4-ALK variant 1 expressed in H3122 lung adenocarcinoma cells that contains the HELP domain in EML4. **(c)** Immunoblot analysis in H2228 cells (whole cell lysate or GST-RBD IP, as indicated) with the indicated antibodies. H2228 cells were treated with 500nM Crizotinib for 1hr. **(d-e)** Cell titer glo viability assays in H3122 and H2228 cells treated with the indicated drug **(d)** crizotinib or **(e)** ceritinib) for 72hr. The bar graph **(f)** depicts IC50 estimation derived from the data. ($n \geq 3$, data are shown $\pm$ SEM for quantitative assays and for immunoblots and immunofluorescence representative of ≥ 3 independent experiments). **(g)** Response of H2228 cell line to trametinib monotherapy using 72h Cell Titer Glo assay. All data shown represent at least 3 independent experiments.

Figure S5

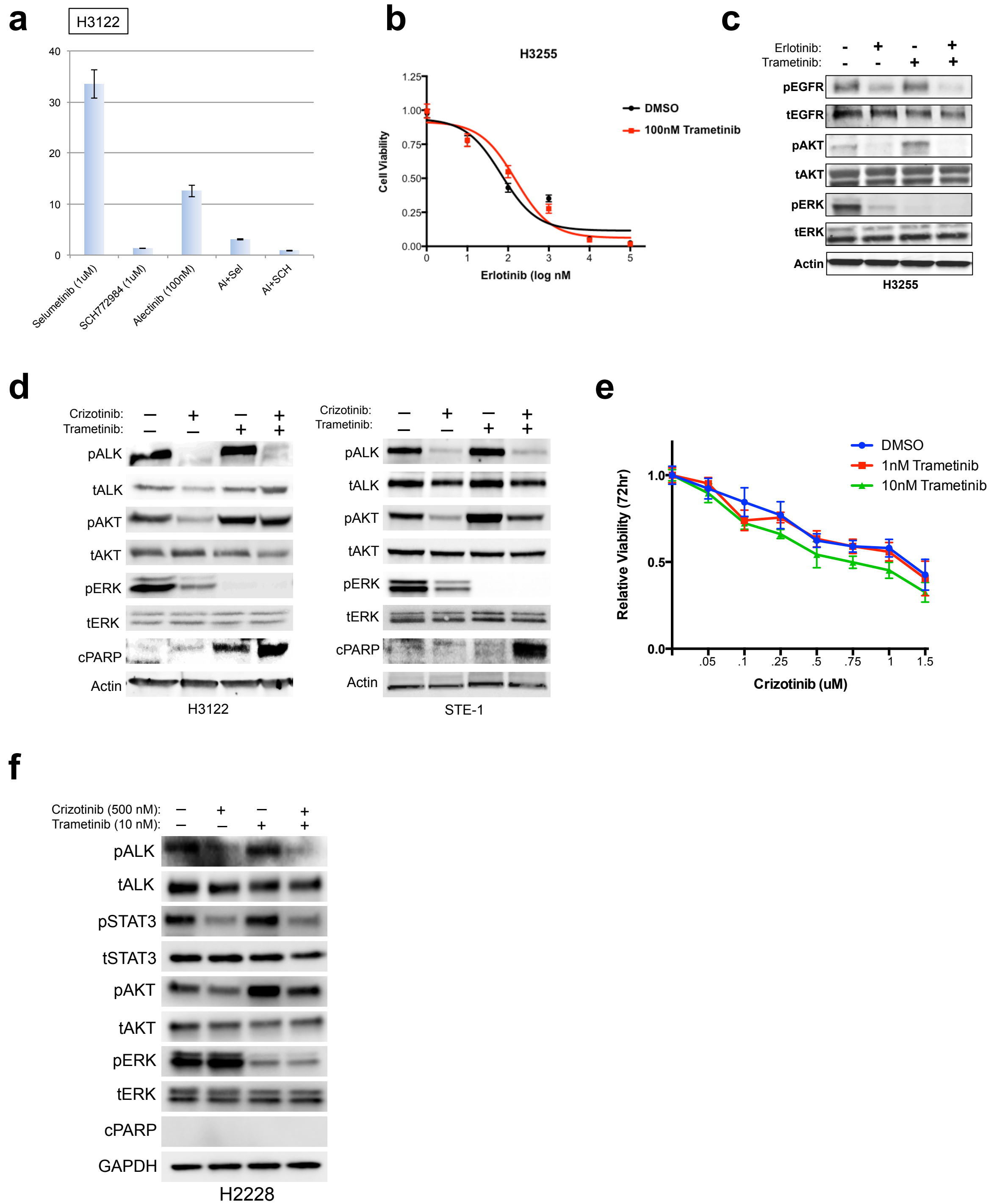

Figure S5. Effects of MAPK pathway inhibition in the lung adenocarcinoma models. (a) Effect of indicated drug treatments on H3122 cell growth. Cells were plated in 6-well plates and cell number was determined 7d after treatment. Values are presented as the percent of cells remaining on d7 compared to vehicle treatment. (**b**) H3255 cells ($EGFR^{L858R}$ lung adenocarcinoma) were treated with erlotinib for 72h with either DMSO or 100nM trametinib in addition. Viability was measured using cell titer glo and values were normalized to DMSO/DMSO control. **(c)** Immunoblot analysis in H3255 cell lysates following treatment for 1h with either erlotinib (100nM), trametinib (100nM), or the combination. **(d)** Immunoblot analysis in H3122 and STE-1 cell lines treated with the indicated drugs (higher dose of Trametinib than Fig. 3d) for 24h. **(e)** H2228 cells treated with sub-maximal doses of trametinib (and combination with Crizotinib) as shown in Fig. 3a-b using 72h Cell Titer Glo assay to assess viability. **(f)** Immunoblot analysis using indicated antibodies on H2228 cells treated with indicated drugs for 24h.

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Figure S6

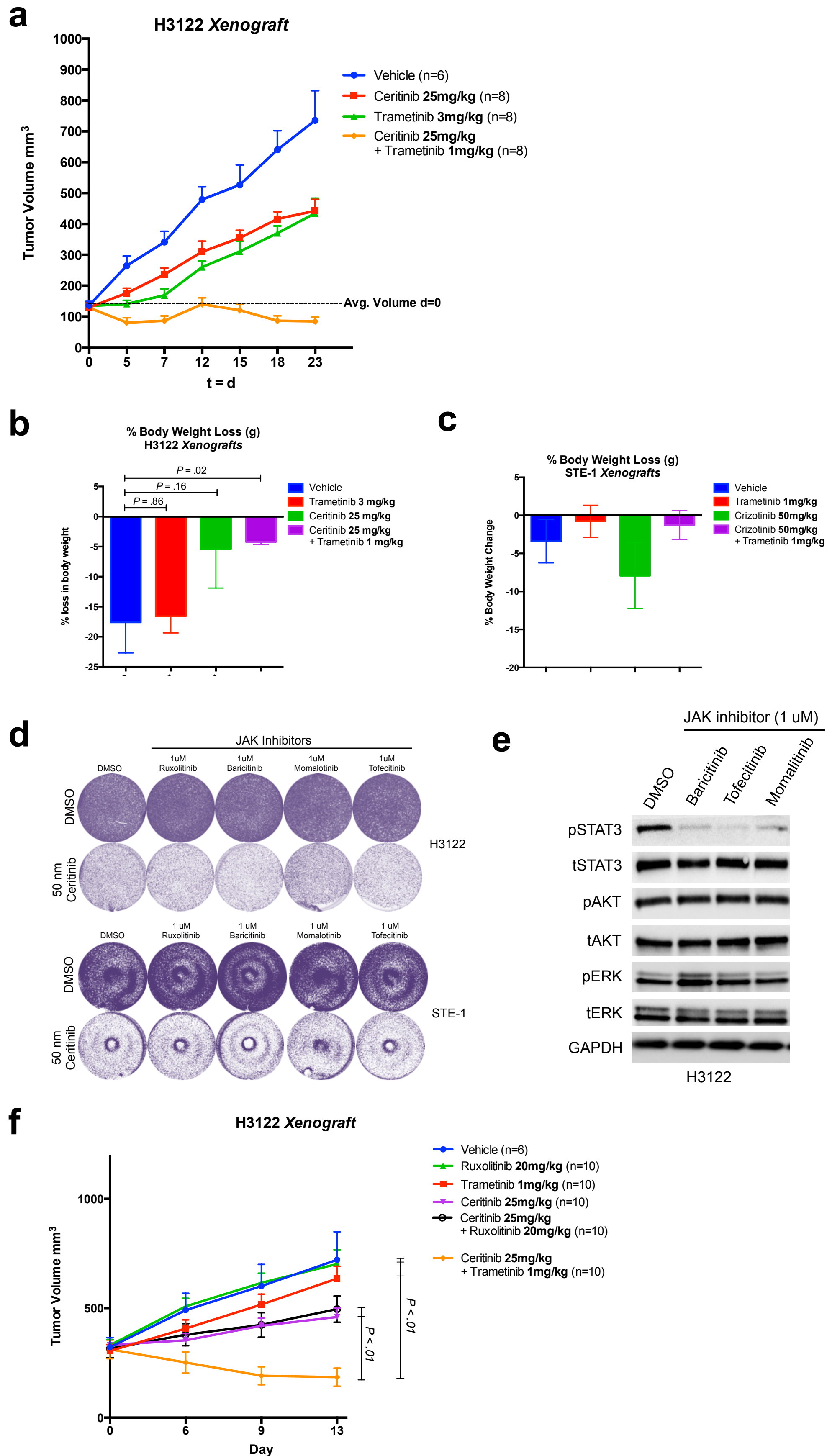
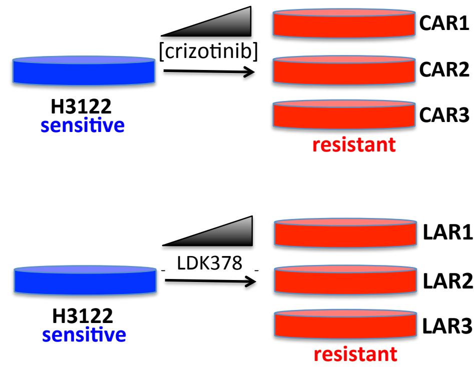


Figure S6. Effects of dual ALK and sub-maximal MEK inhibition in the *in vivo* EML4-ALK lung adenocarcinoma models. (a) H3122 xenografts from Fig. 3e. Mice were treated with indicated concentrations of drug once daily. **(b-c)** Percent loss in body weight (g) of nude mice engrafted with **(b)** H3122 or **(c)** STE-1 cells and treated with indicated regimens. Values represent change in weight from study endpoint (d=23 or d=31) from baseline (d=0). *P* value was determined using unpaired t-test between treatment group and vehicle. ($n \geq 3$, data are shown $\pm$ SEM for quantitative assays and for immunoblots representative of ≥ 3 independent experiments). (**d**) Crystal violet cell growth assays in the indicated cells treated with the indicated agents, including JAK inhibitors as shown. All data shown represent at least 3 independent experiments. **(e)** Immunoblot analysis of H3122 cells treated with JAK inhibitors (24h) from (**d**). **(f)** Nude mice engrafted with H3122 cells were treated with indicated treatment regimens for 13d, and tumor size (mm^3) was measured.

Figure S7

a



b

Cell Line	Crizotinib IC50 (nM)	Ceritinib IC50 (nM)	Growth Rate/H3122
H3122	220	110	1.00 ± .02
CAR1	2800	1050	1.29 ± .06
CAR2	4200	1600	0.79 ± .04
CAR3	4100	1550	0.75 ± .06
LAR1	2100	>2000	0.95 ± .10
LAR2	2700	1950	1.10 ± .06
LAR3	1600	>2000	1.20 ± .16

c

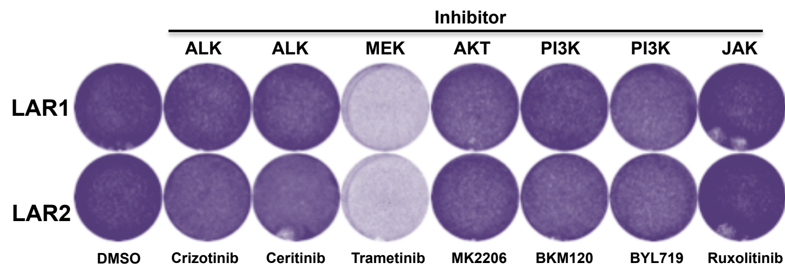
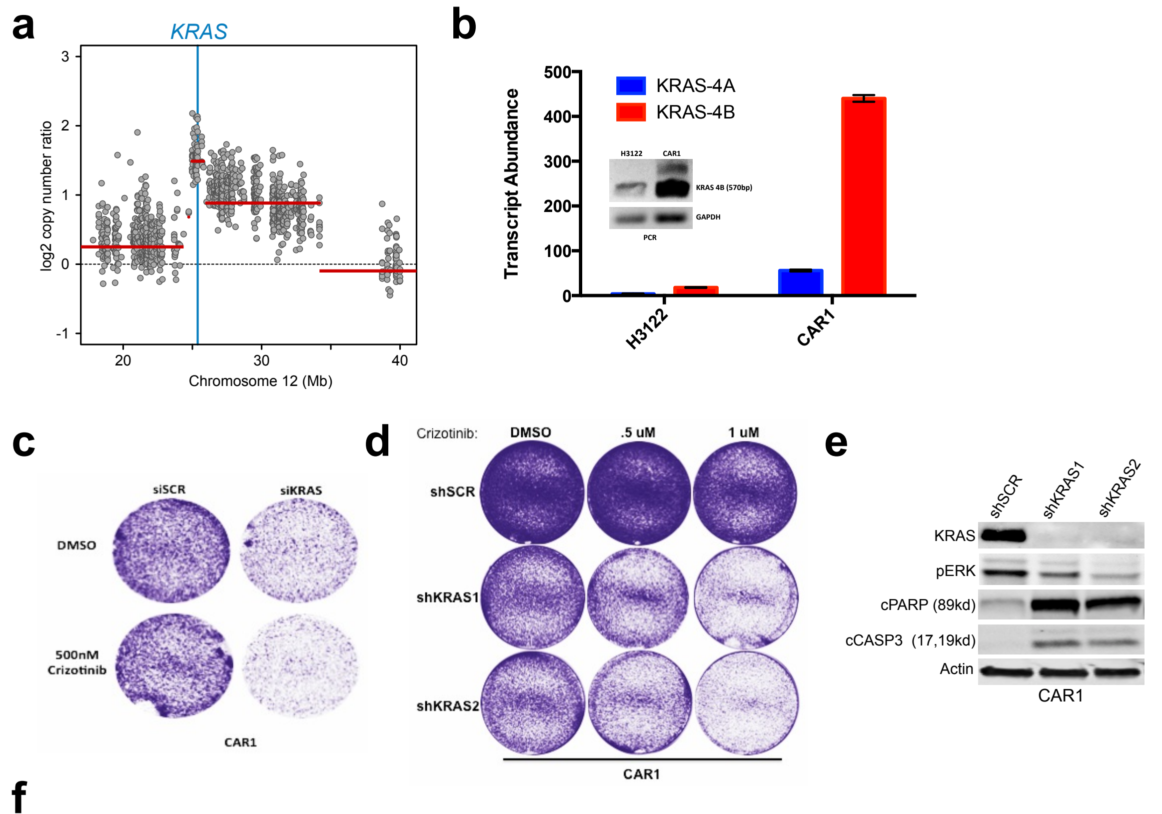


Figure S7. ALK inhibitor resistant EML4-ALK lung adenocarcinoma cell lines derived and studied. (**a**) Schematic representation of the protocol to derive ALK TKI isogenic resistant cell lines. H3122 were treated with escalating doses of crizotinib (100nM, 250nM, 500nM, 1000nM) or ceritinib (10nM, 50nM, 100nM, 200nM) for 90 days. Resistant lines were maintained at either 1uM crizotinib (CAR) or 200nM ceritinib (LAR). (**b**) Crizotinib and ceritinib IC50 values for CAR and LAR lines. Growth rates of CAR and LAR lines (in the presence of either 1uM crizotinib or 200nM ceritinib, respectively) are depicted relative to parental H3122 growth rate. (**c**) LAR1 and LAR2 cells were plated in 6-well plates and treated with indicated concentration of indicated inhibitors. Cells were fixed at day 5 and stained with crystal violet.). ($n \geq 3$, data are shown $\pm$ SEM for quantitative assays and for crystal violoet assays representative of ≥ 3 independent experiments)

Figure S8



f

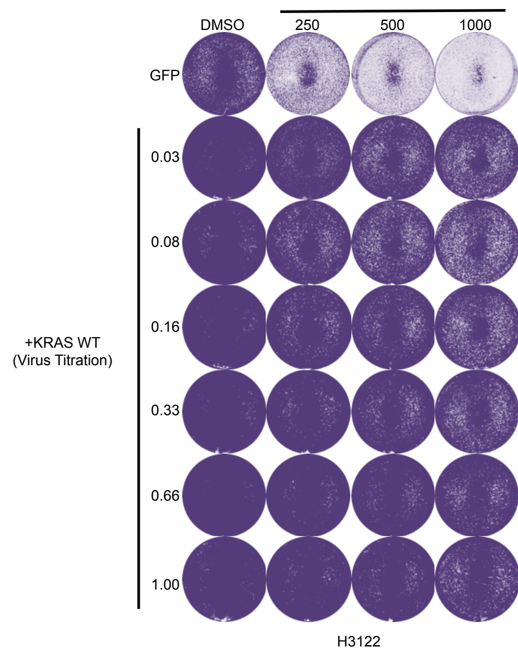
Patient	Age	Sex	ALK inhibitor	ALK resistance mutation	RAS/RAF/MEK mutation	KRAS Copy Number Alteration
1	27	F	Ceritinib	ALK F1174C	None[a]	Negative
2	70	M	Crizotinib	None	None[b]	Amplification[d]
3	49	F	Crizotinib	ALK L1196M	None[b]	Duplication[c]
4	42	F	Crizotinib	ALK CNG	None[a]	Duplication[c]
5	40	M	Crizotinib, AP26113	None	Not tested	Duplication[c]
6	54	M	Crizotinib	None	None[a]	Duplication[c]
7	62	F	Crizotinib	ALK G1269A	None[a]	Duplication[c]
8	41	M	Crizotinib	None	None[a]	Negative
9	41	F	Crizotinib	None	None[a]	Negative
10	75	M	Crizotinib	EGFRdel19	None[a]	Duplication[c]
11	52	F	Crizotinib, Ceritinib	None	None[a]	Negative
12	51	M	Crizotinib, AP26113	ALK CNG	None[a]	Negative
13	53	F	Crizotinib	None	None[e]	Amplification[d]
14	41	F	Crizotinib	None	None[e]	Amplification[d]
15	44	M	Crizotinib	None	None[b]	Duplication[c]

[a]KRAS, NRAS, BRAF, MEK by SNaPshot
[b]by direct sequencing for KRAS, NRAS, BRAF, MEK1/2
[c]atypical KRAS FISH pattern including doublets in post-treatment biopsy
[d]KRAS/CEP12 ratio > 2.2 in post-treatment tumor cells
[e]by direct sequencing for NRAS, BRAF, MEK1/2

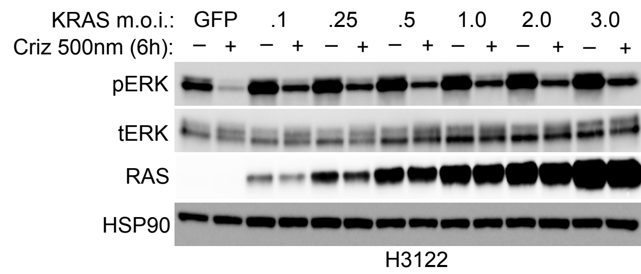
Figure S8. KRAS^{WT} genetic upregulation drives ALK inhibitor resistance. (**a**) Exome analysis of CAR1 reveals a focal amplification on chromosome 12 spanning KRAS^{WT}. (**b**) qPCR using primers designed for KRAS-4A and KRAS-4B transcripts in H3122 and CAR1. Inset shows representative semi-quantitative PCR results. **(c)** Growth inhibition in response to KRAS siRNA transfection +/- crizotinib in CAR1. Cells were transfected for 48h, and treated with indicated concentration of crizotinib for 96h, then fixed and stained with crystal violet. (**d**) CAR1 cells were transduced with two independent shRNAs targeting KRAS. Cells were then plated and exposed to the indicated doses of crizotinib and fixed and stained at 5 days after treatment. (**e**) Immunoblot analysis with the indicated antibodies in CAR1 cell lysates after introduction of either shRNA-Scramble or shKRAS1, or shKRAS2. (**f**) Table summarizing ALK fusion lung adenocarcinoma patient characteristics (n=15). All patients were treated with the indicated ALK TKI and tumor biopsies were obtained after resistance (where available, the corresponding pre-treatment tumor sample was also analyzed, patients #2, #13, #14). The presence of ALK, RAS, RAF, or other mutations are noted along with KRAS copy number alteration status (where sufficient tumor sample was available for such analysis).

Figure S9

a



b



c

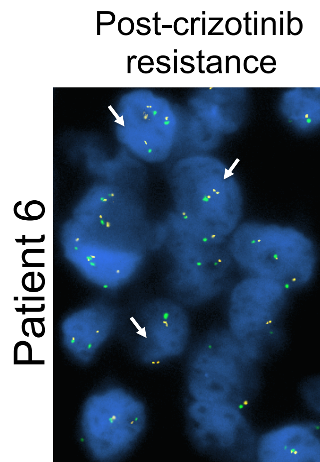
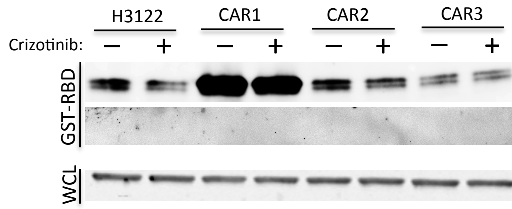


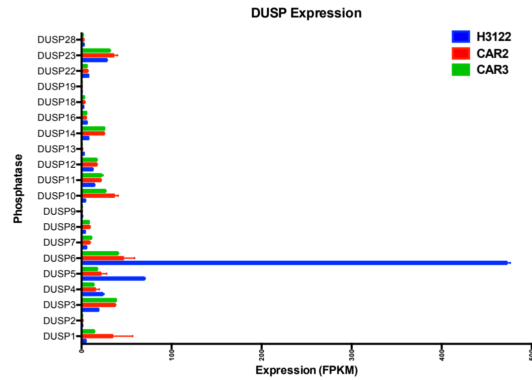
Figure S9. Effects of relatively low levels of $KRAS^{WT}$ expression in EML4-ALK lung adenocarcinoma cell lines. (**a-b**) H3122 cells were transduced with increasing viral titrations to achieve differential levels of $KRAS^{WT}$ expression. These isogenic cells were then assayed for Crizotinib response using **(a)** Crystal Violet assay and **(b)** immunoblot analysis with the indicated antibodies to assess downstream signaling (immunoblots and crystal violet assays representative of $\geq$3 independent experiments). **(c)** Representative image of KRAS gene duplication events as measured by KRAS FISH in ALK fusion positive patient tumor biopsy with acquired resistance to ALK TKI (the ALK inhibitor resistant tumor specimen from patient #6 is shown). White arrowheads indicate tumor cells with KRAS copy number gain events.

Figure S10

a



b



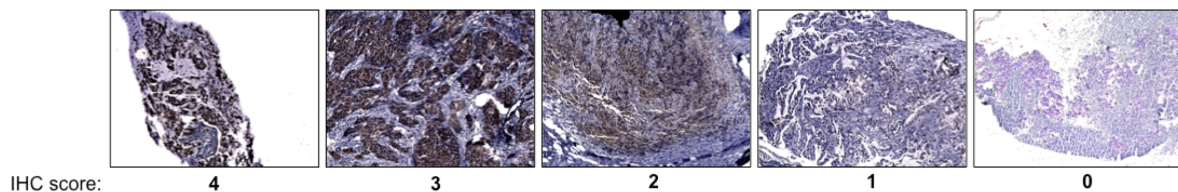
c

	Patient	Age	Sex	ALK inhibitor	DUSP6 IHC Score (0-4): Pre-Treatment	DUSP6 IHC Score (0-4): Post-Crizotinib Resistance	Post-Crizotinib Treatment, Response
*	13	53	F	Crizotinib	4	2[a]	*ASP3026, 4m
*	14	41	F	Crizotinib	3	2[a]	*ASP3026, 4m
*	15	44	M	Crizotinib	2	0[c]	*ASP3026, 4.5m
*	16	40	M	Crizotinib	3	1[d]	MPDL3280A, 16m
	17	62	F	Crizotinib	na	2[d]	na
	18	47	F	Crizotinib	na	2[d]	*Ceritinib, 8m
*	19	30	M	Crizotinib	2	2[d]	na
	20	60	F	Crizotinib	na	0[b]	*Ceritinib, 2m
*	21	65	M	Crizotinib	3	1[d]	*Chemotherapy, 4m
	22	76	F	Crizotinib	2	na	na
	23	49	M	Crizotinib	1	na	na
	24	57	M	Crizotinib	2	na	na
	25	54	F	Crizotinib	3	na	na
	26	68	M	Crizotinib	2	na	na
	27	42	F	Crizotinib	4	na	na
	28	45	F	Crizotinib	1	na	na
	29	77	F	Crizotinib	2	na	na
	30	45	M	Crizotinib	na	0[b]	No ALK TKI
	31	60	M	Crizotinib	3	na	na

* = matched samples * = ALK TKI (treatment post-Crizotinib) * Carboplatin/Pemetrexed

[a]KRAS CNG positive
[b]KRAS CNG negative
[c]KRAS doublets/duplication
[d]KRAS CNG not tested

d



IHC score: 4 3 2 1 0

Figure S10. RAS-MAPK pathway activation in ALK inhibitor resistant EML4-ALK lung adenocarcinoma. (**a**) GST-RBD pull down assays in H3122 and CAR lines showing RAS-GTP levels -/+ 500nM crizotinib (6hr). (**b**) mRNA expression levels (fpkm) of DUSP family members in H3122, CAR2, and CAR3. (**c**) Table showing the patient cohort assayed for DUSP6 using IHC (score 0-4). Matched pairs are indicated with a blue asterisk. "na" indicates no available tissue for analysis. Additionally, post-Crizotinib treatments are indicated where available. **(d)** Examples of DUSP6 IHC scores used in **(c)**. A score of "0" represents no apparent DUSP6 staining in tumor cells, "1" represents low levels of identifiable staining in any tumor cell populations. "2" represents moderate DUSP6 staining intensity in tumor cells. "3" represents high, and "4" represents very high DUSP6 staining in tumor cells. Scoring was performed by 3 blinded individuals and consensus scores are presented.

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