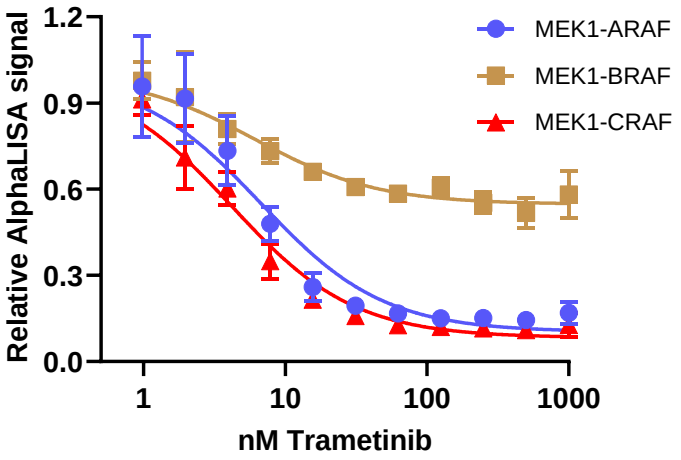
Supplementary Figure S1

Related to Figure 1

A

	NST-628	Trametinib	Avutometinib
pMEK IC_{50} (nM) HCT116 ($KRAS^{G13D}$)	0.31	1.9	7.2
pMEK IC_{50} (nM) A549 ($KRAS^{G12S}$)	0.26	1.4	3.8
pERK IC_{50} (nM) HCT116 ($KRAS^{G13D}$)	0.1	0.82	0.22
pERK IC_{50} (nM) A549 ($KRAS^{G12S}$)	0.11	1.2	0.18

B

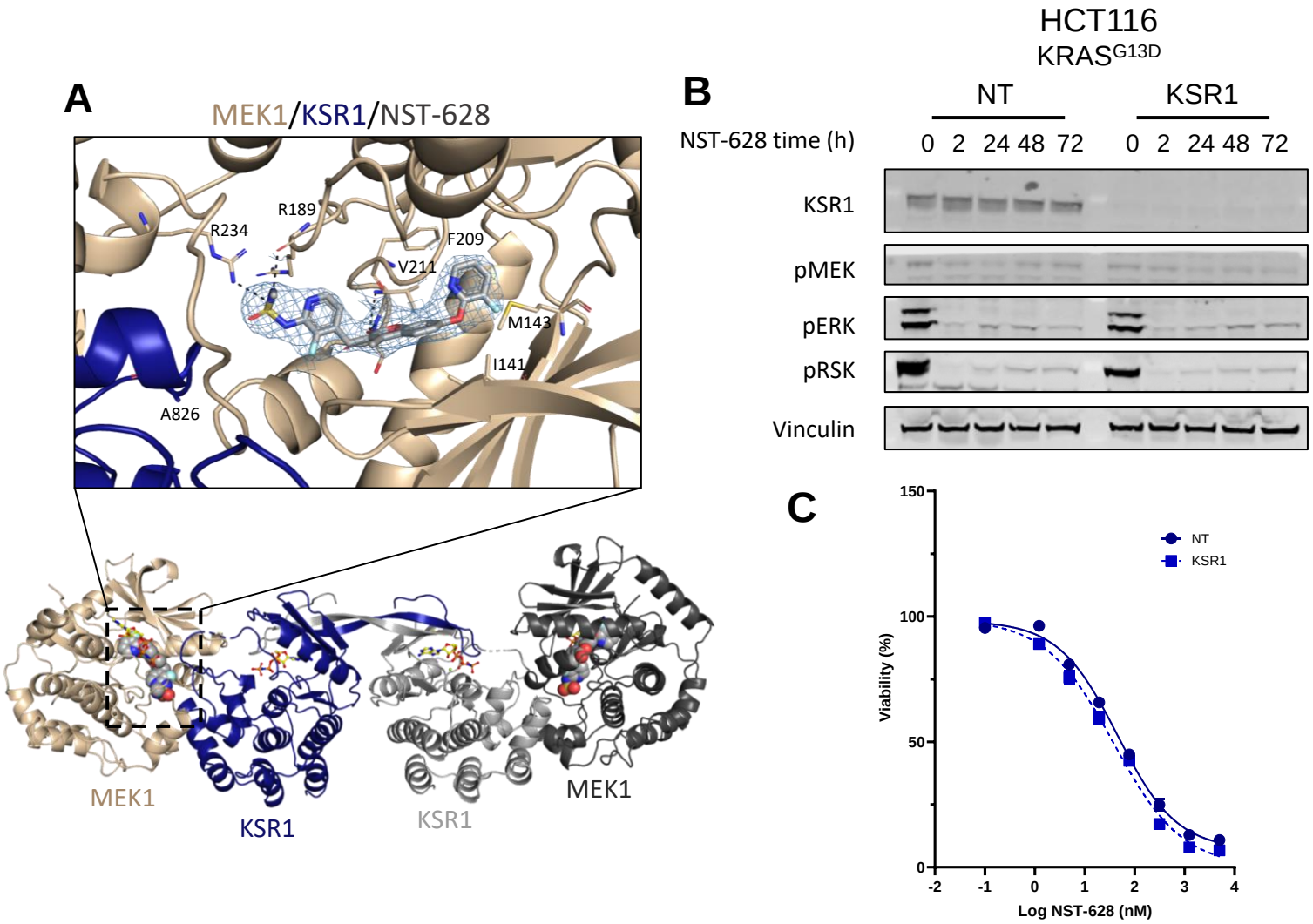


	IC50 (nM)	Top
MEK1-ARAF	6.7	0.13
MEK1-BRAF	6.0	0.51
MEK1-CRAF	4.1	0.08

Supplementary Figure S1. (A) HCT116 and A549 cells were treated with a dose response of NST-628, trametinib, or avutometinib for 2 h and pathway inhibition was measured by phospho-MEK AlphaLISA or phospho-ERK HTRF. (B) MEK1-RAF complex formation monitored by AlphaLISA protein-protein interaction assays after treatment with various concentrations of trametinib for 30 min at RT.

Supplementary Figure S2

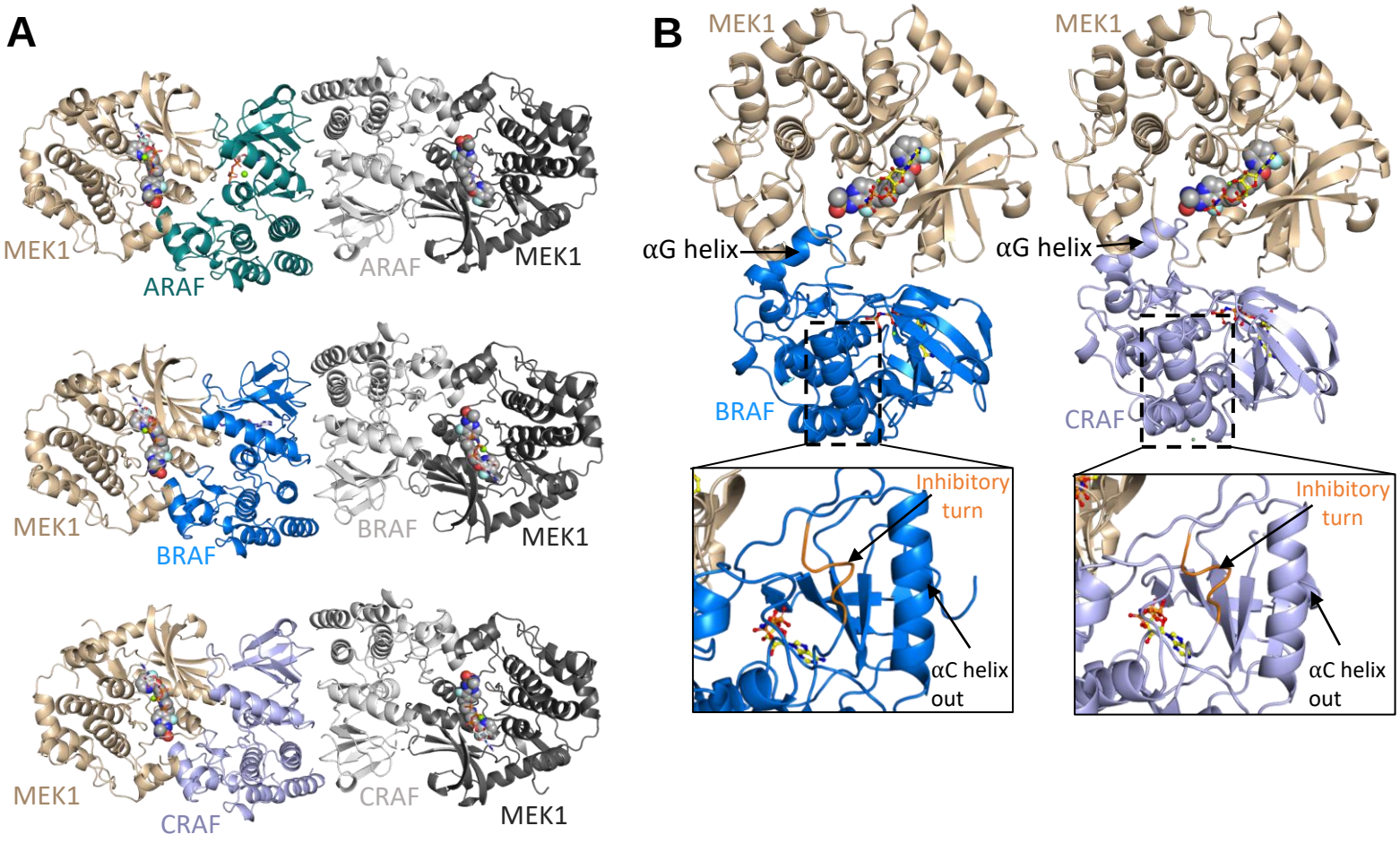
Related to Figure 2



Supplementary Figure S2. (A) Overview of the MEK1-KSR1 heterotetramer crystal structure (2.81Å resolution) bound to NST-628 (shown as spheres). The KSR1 dimer observed in the structure is mediated by an N-lobe domain swap. Inset shows electron density for NST-628 (blue mesh) with key interactions highlighted by black dashes. (B) Western blots and (C) 72h proliferation (CellTiter-glo) of KSR1 siRNA knockdown HCT16 cells treated with NST-628. (B) Blot analysis was performed to confirm KSR1 loss and monitor phospho-MEK, phopsho-ERK, phospho-RSK, and vinculin as a loading control after 2-72 h of 10 nM NST-628 treatment (C) HCT116 NT and KSR1 knockdown cell lines were treated with a dose response of NST-628 for 72 h and viability was assessed by CellTiter-Glo.

Supplementary Figure S3

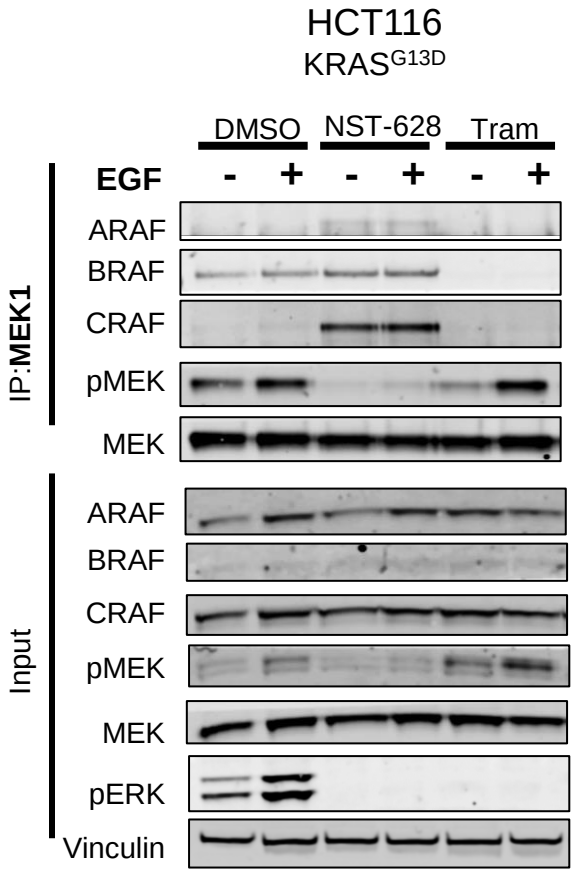
Related to Figure 2



Supplementary Figure S3. (A) Arrangement of the MEK1-RAF heterotetramers observed in crystal structures with active RAF conformation and NST-628 (shown as spheres). Symmetry related MEK1-RAF complexes are shown in grey. (B) Overview of the crystal structures of MEK1-BRAF (3.68Å resolution) and MEK1-CRAF (2.41Å resolution) with NST-628 (shown as spheres) and inactive RAF. Insets show the outward conformation of the RAF αC-helix supported by the inhibitory turn (orange).

Supplementary Figure S4

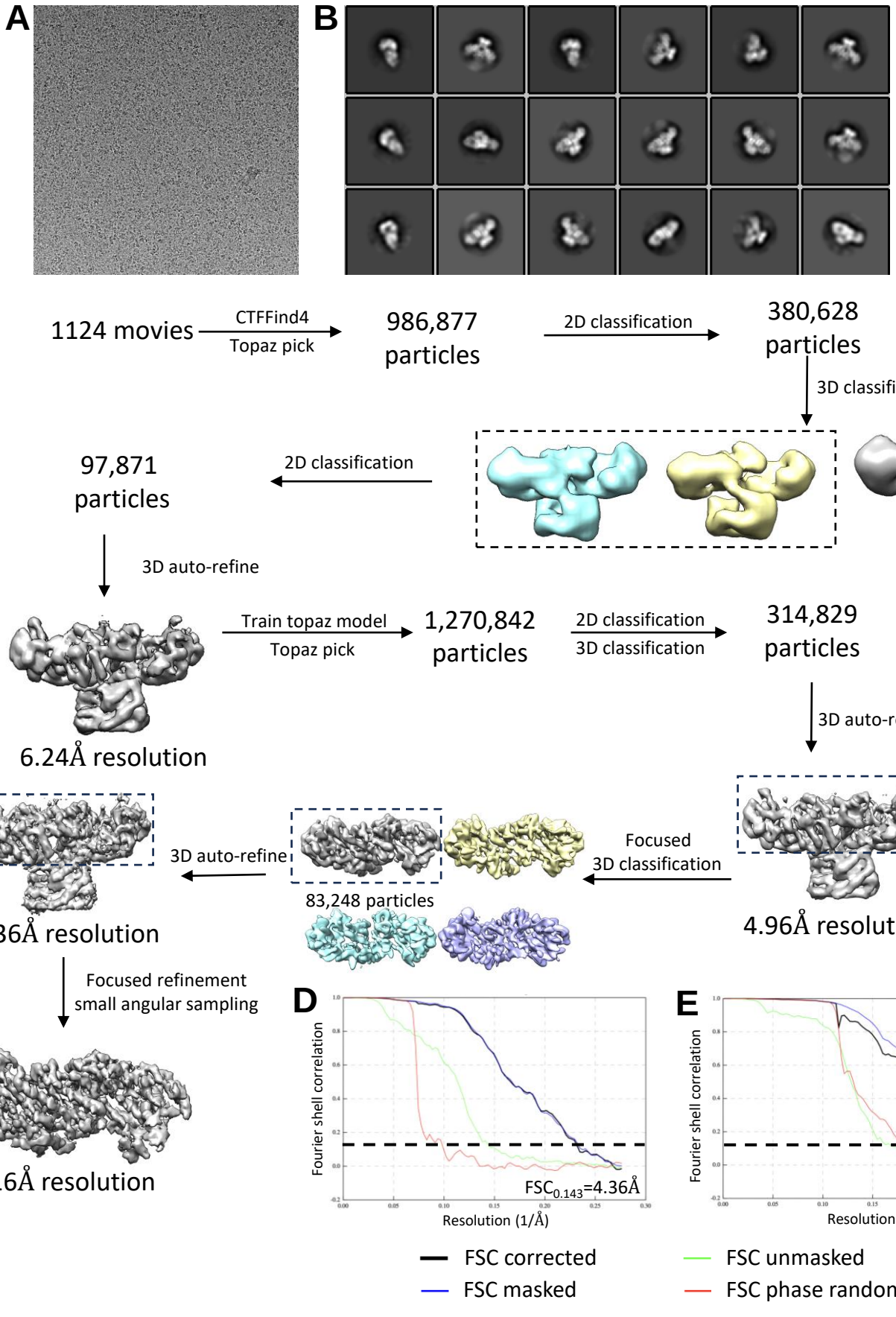
Related to Figure 2



Supplementary Figure S4. HCT116 cells treated with 100 nM NST-628 or trametinib for 2 h and then stimulated with EGF (100 ng/ml) for 10 minutes. Samples were subjected to a MEK1 immunoprecipitation and blot analysis was performed for ARAF, BRAF, CRAF, phospho-MEK, MEK1, phospho-ERK and vinculin as a loading control

Supplementary Figure S5

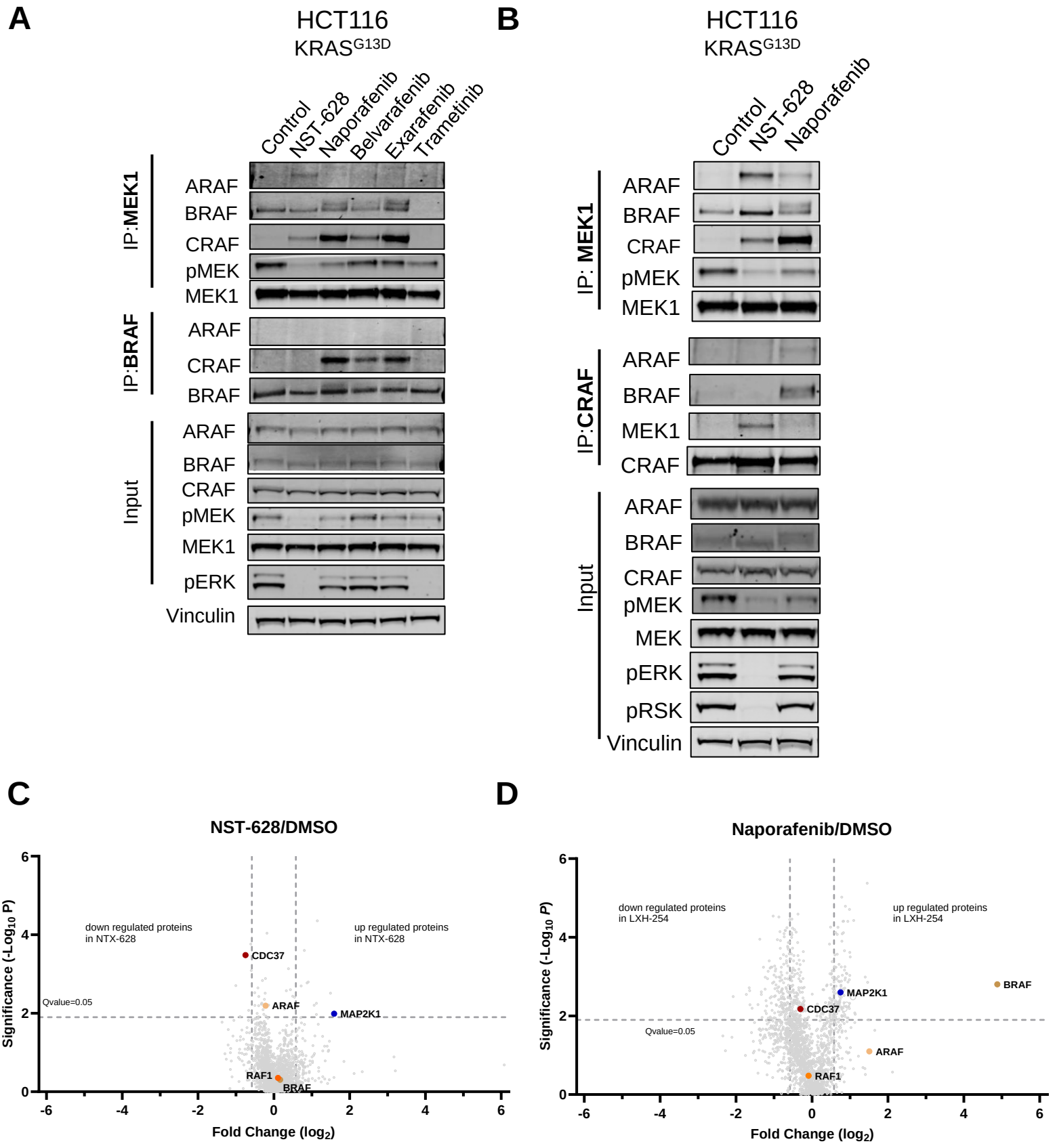
Related to Figure 2



Supplementary Figure S5. A, Example micrograph from the MEK1-CRAF-14-3-3-NST-628 dataset. B, Example 2D classes corresponding to $MEK1_2$-$CRAF_2$-14-3-3_2 with NST-628. C, Image processing workflow for $MEK1_2$-$CRAF_2$-14-3-3_2 with NST-628. FSC curves for the 4.36Å resolution consensus refinement (D) and 4.16Å resolution focused refinement (E). Black dashed lines represent $FSC_{0.143}$.

Supplementary Figure S6

Related to Figure 3

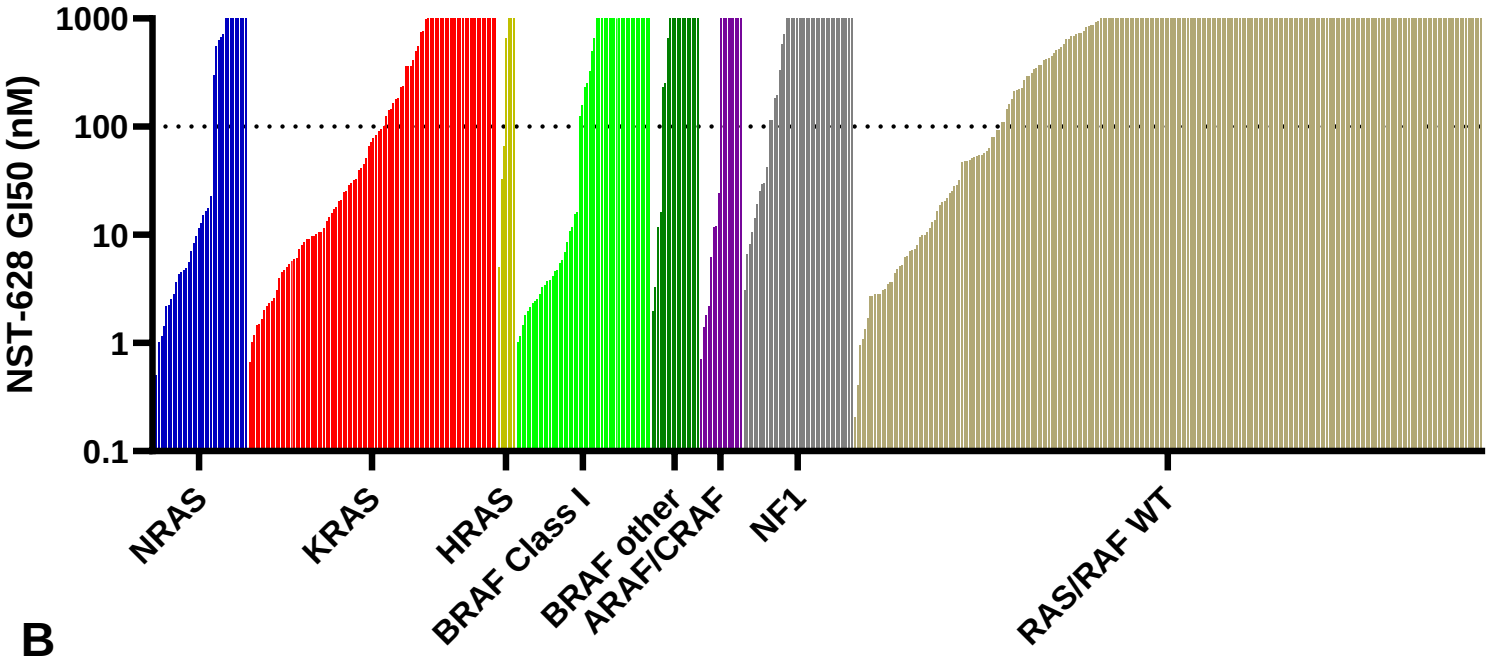


Supplementary Figure S6. (A) MEK1 or BRAF immunoprecipitation in HCT116 cells treated with 100 nM NST-628, naporafenib, belvarafenib, exarafenib or trametinib for 2 h and blot analysis was performed for ARAF, BRAF, CRAF, phospho-MEK, MEK1, phospho-ERK and vinculin as a loading control. (B) MEK1 or CRAF immunoprecipitation in HCT116 cells treated with 100 nM NST-628 or naporafenib for 2 h and blot analysis was performed for ARAF, BRAF, CRAF, phospho-MEK, MEK1, phospho-ERK and vinculin as a loading control CRAF immunoprecipitation and mass spectrometry of cells treated with (C) NST-628 or (D) naporafenib. Samples were normalized to DMSO for expression of CRAF interacting proteins.

Supplementary Figure S7

Related to Figure 4

A



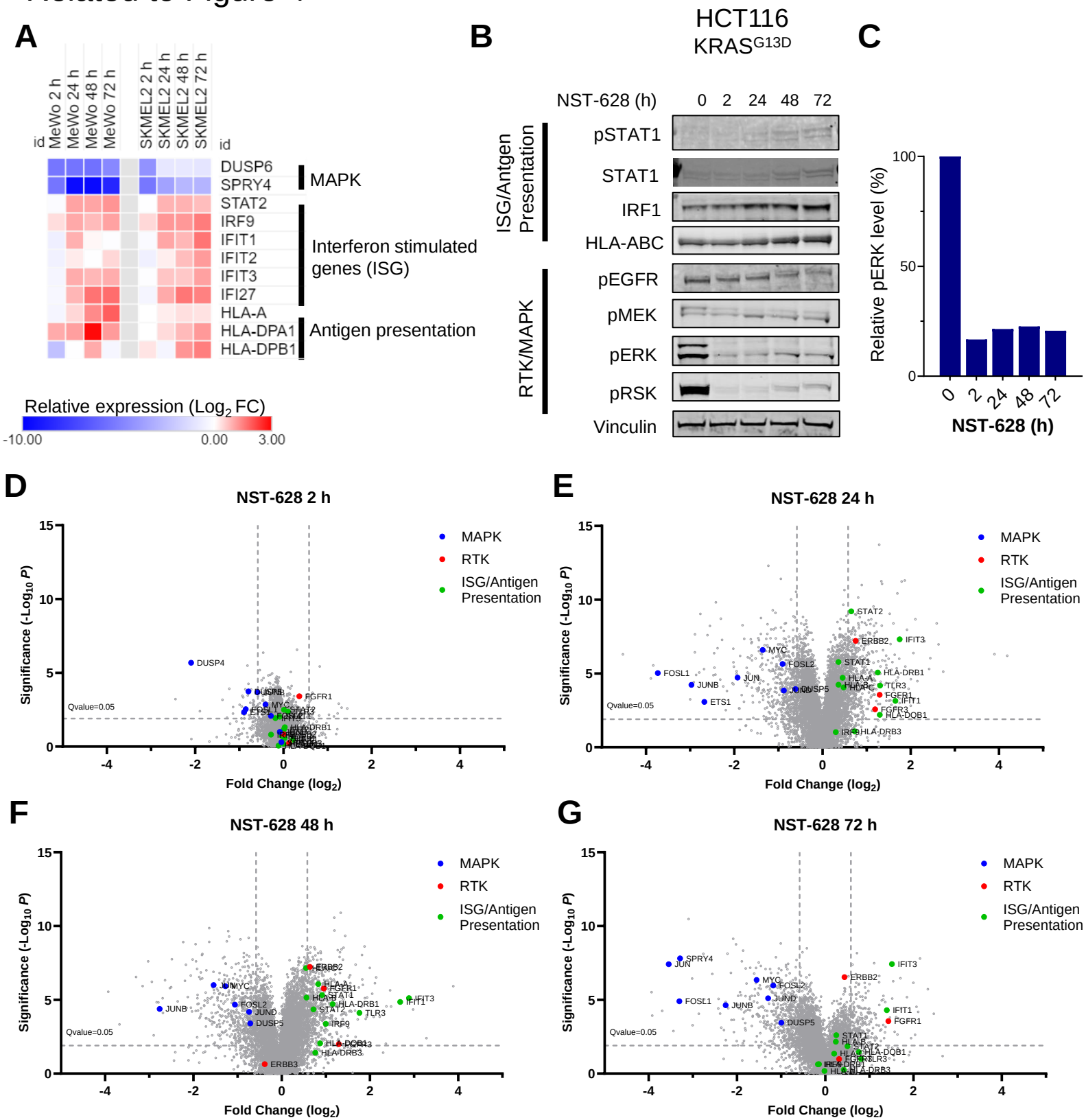
B

	NRAS	KRAS	BRAF Class I	BRAF other	HRAS	NF1	ARAF/CRAF	RAS/RAF WT
GI50 ≤100 nM	25	60	17	6	4	11	9	67
Total models	38	107	30	19	8	45	17	289
Sensitive (%)	65.8	56.1	56.7	31.6	50	24.4	52.9	23.2

Supplementary Figure S7 (A) OMNI cell line panel treated was treated with a dose-response of NST-628 for between 3 and 7 days and viability was measured by CellTiter-Glo. (B) Response rates in each mutational background were calculated for models with a GI50 of ≤ 100 nM.

Supplementary Figure S8

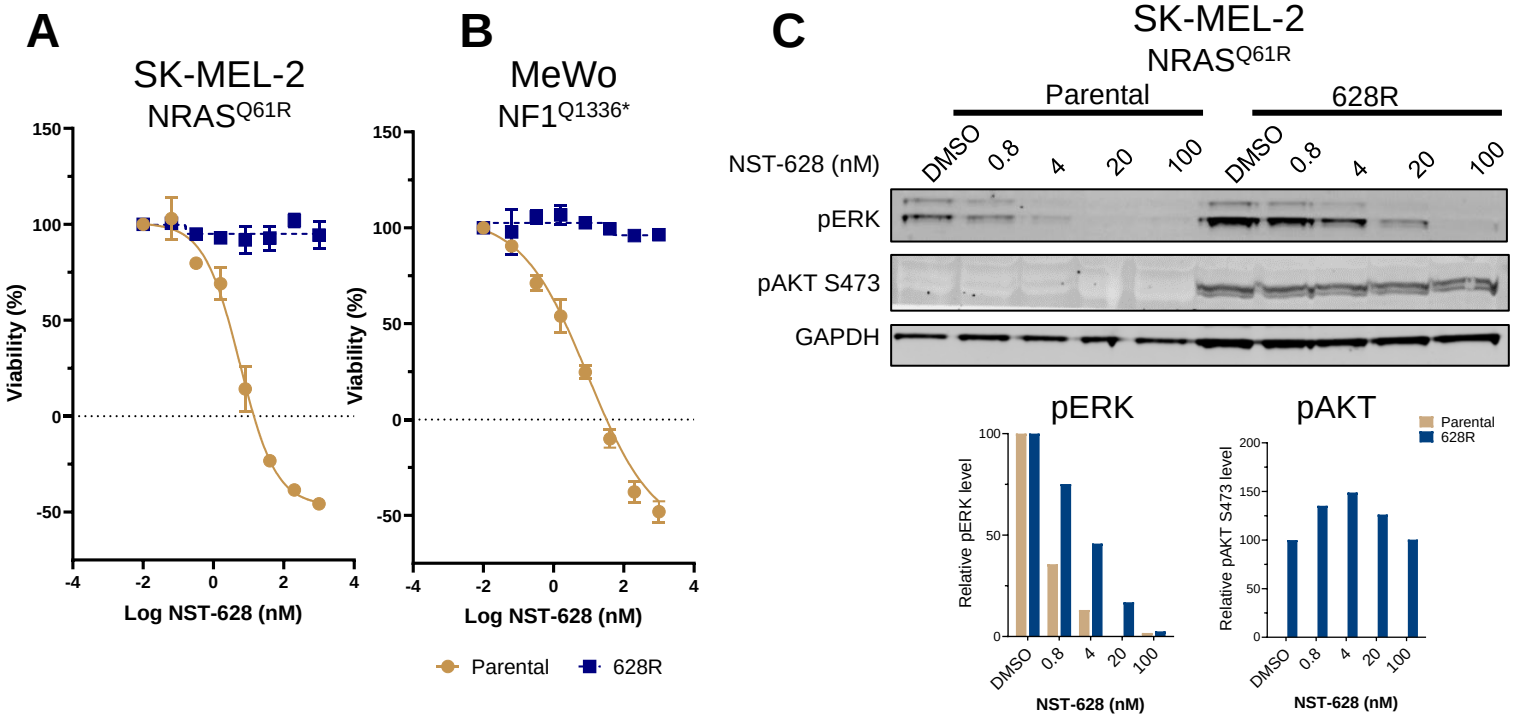
Related to Figure 4

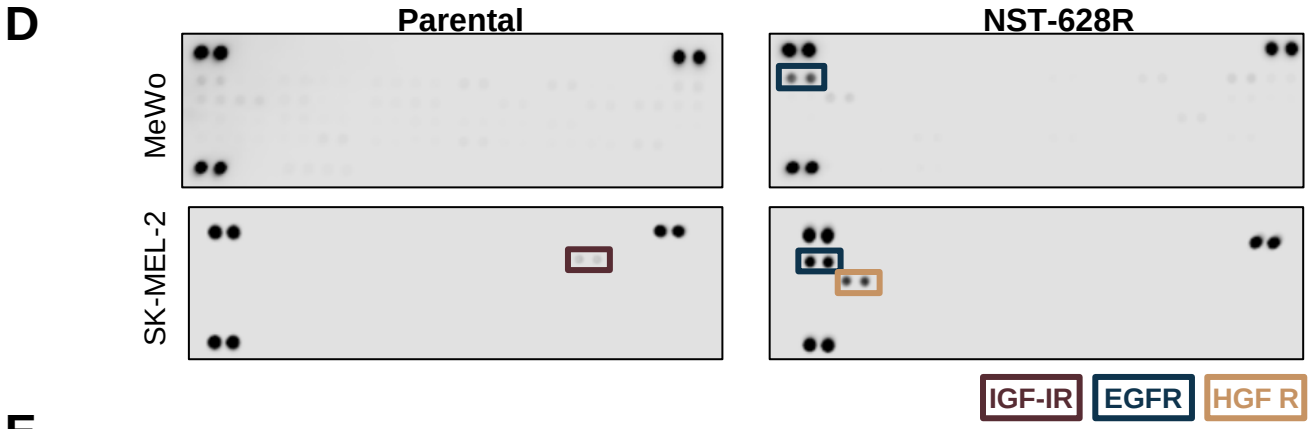


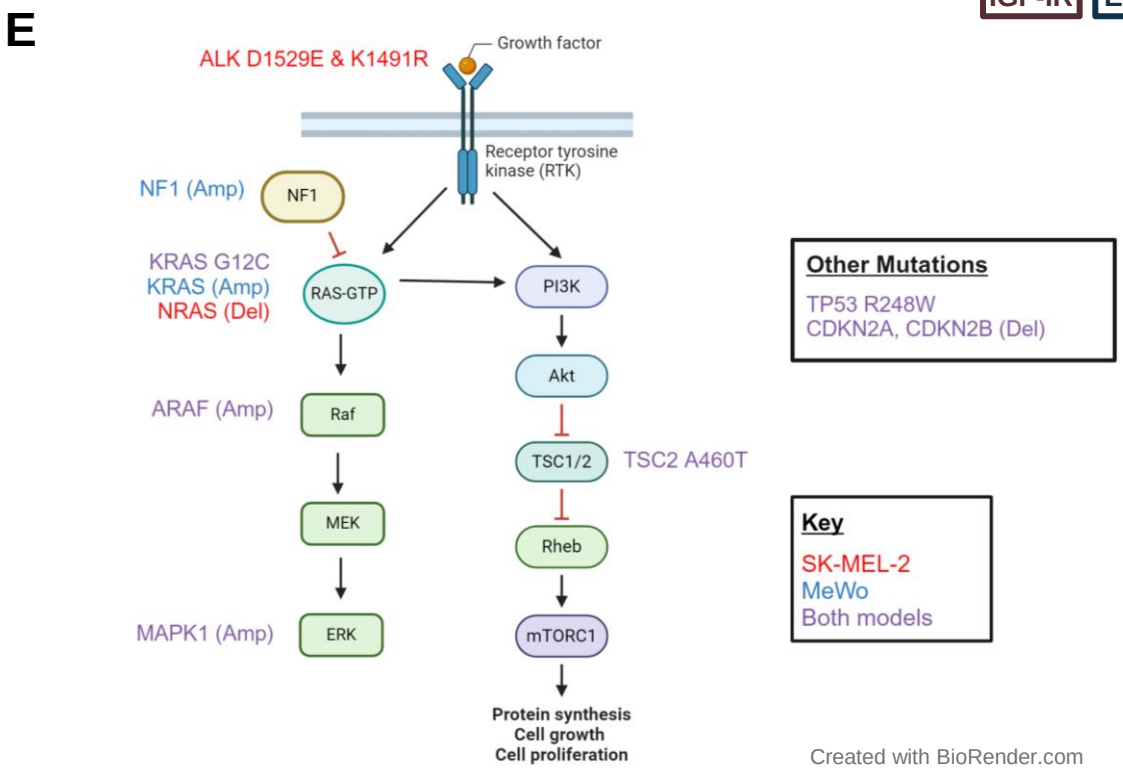
Supplementary Figure S8 (A) Differentially expressed genes (RNAseq) of MeWo and SK-MEL-2 cell lines treated with NST-628 for 2-72 h (B) HCT116 cells treated with 20 nM NST-628 for 2-72 h. A blot analysis was performed for phospho(Y701)- and total STAT1, IRF1, HLA-ABC, phospho-EGFR (Y1068) phospho-MEK, phospho-ERK, phospho-RSK and vinculin as a loading control. (C) Densitometry analysis of phospho-ERK in B. (D-G) Global proteomics in the HCT116 cell line after 2, 24, 48, or 72 h of NST-628 treatment

Supplementary Figure S9

Related to Figure 4







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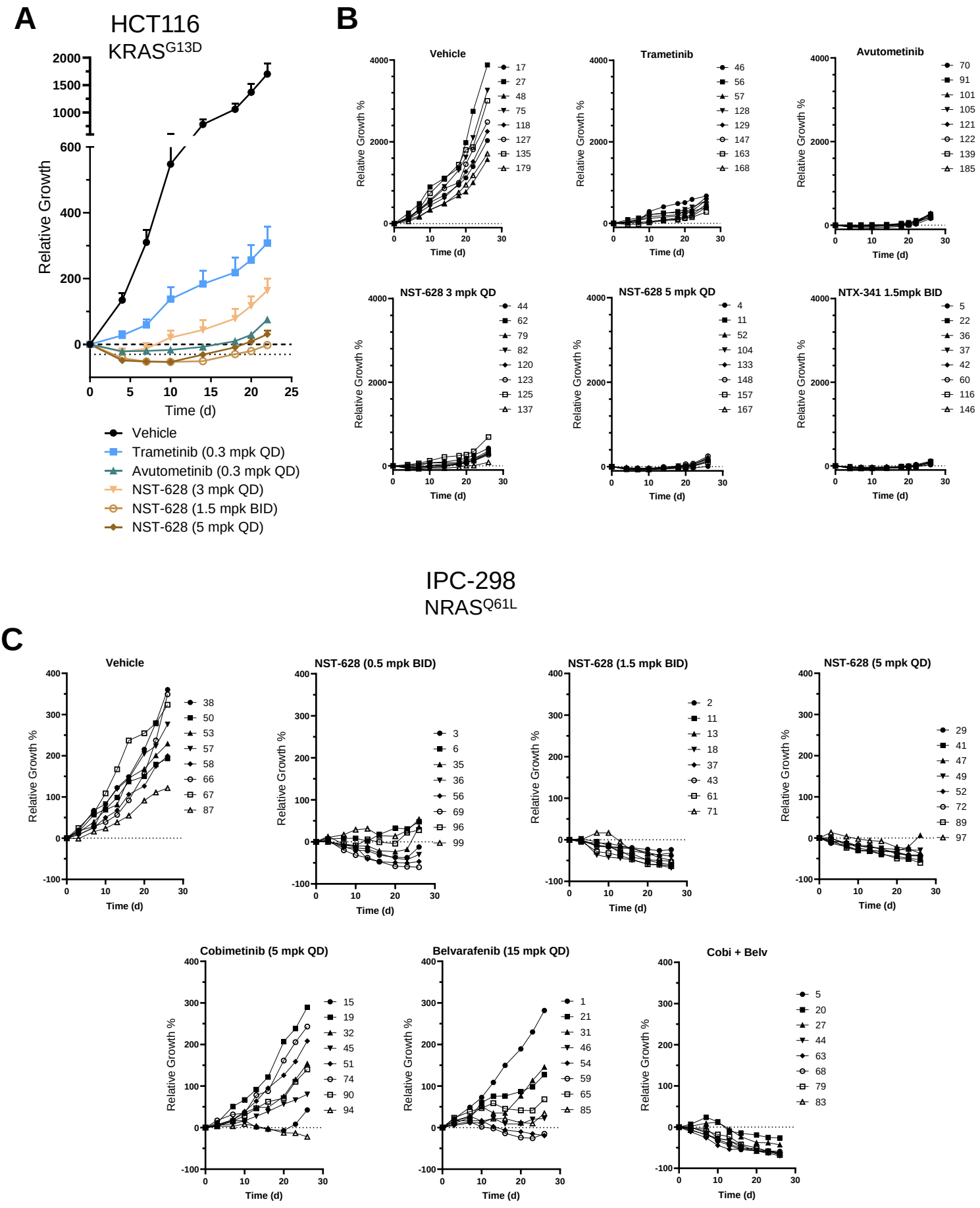
Supplementary Figure S9 (A) SK-MEL-2 and (B) MeWo parental and NST-628 resistant cell lines were treated with a dose response of NST-628 for 72 h and viability was assessed by CellTiter-Glo. (C) SK-MEL-2 parental and NST-628 resistant cell lines were treated with indicated concentrations of NST-628 (4-100 nM) for 2 h and blot analysis was performed phospho-ERK, phospho-AKT, and vinculin as a loading control. Densitometry analysis was performed for phospho-ERK and phospho-AKT. (D) RTK array analysis for MeWo and SK-MEL-2 parental and NST-628 resistant cell lines. (E) Summary of whole exome sequencing analysis for NST-628 resistant MeWo and SK-MEL-2 cell lines compared to parental cell lines. Genes are categorized as mutant in SK-MEL-2 (red), MeWo (blue), both cell lines (purple).

Supplementary Figure S10

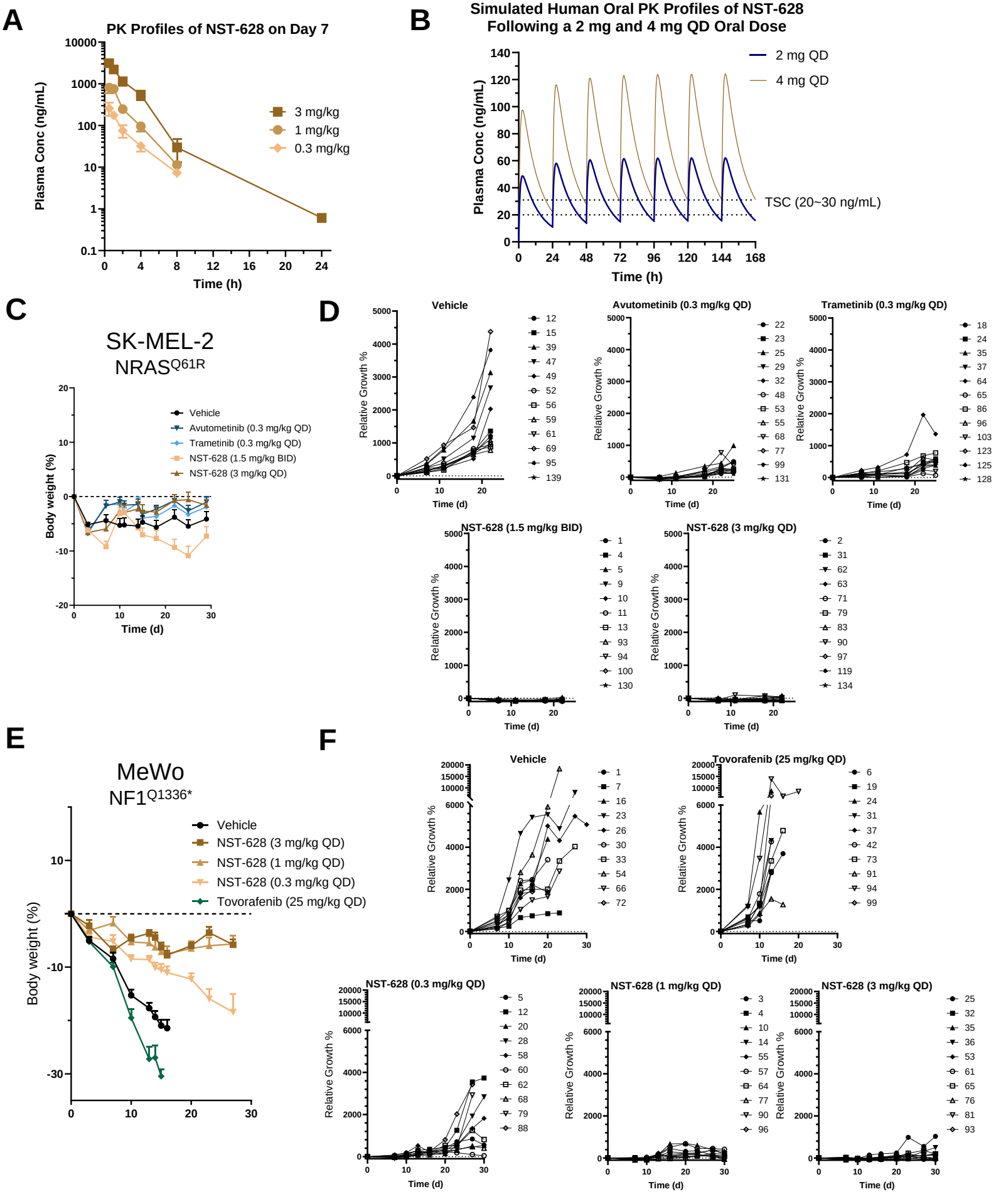
Related to Figure 5



Supplementary Figure S10. (A) Tumor volume of HCT116 tumors treated with 0.3 mg/kg QD trametinib, 0.3 mg/kg QD avutometinib, 3 mg/kg QD, 5 mg/kg QD, or 1.5 mg/kg BID NST-628; tumors are normalized to D0 starting volume (B) Individual tumor volumes of mice from panel A (C) Individual IPC-298 tumors from Figure 5 treated with 0.5 mg/kg BID, 1.5 mg/kg BID, or 5 mg/kg QD NST-628, cobimetinib (5 mg/kg QD), belvarafenib (15mg/kg QD), or a combination of cobimetinib (5 mg/kg daily) and belvarafenib (15 mg/kg)

Supplementary Figure S11

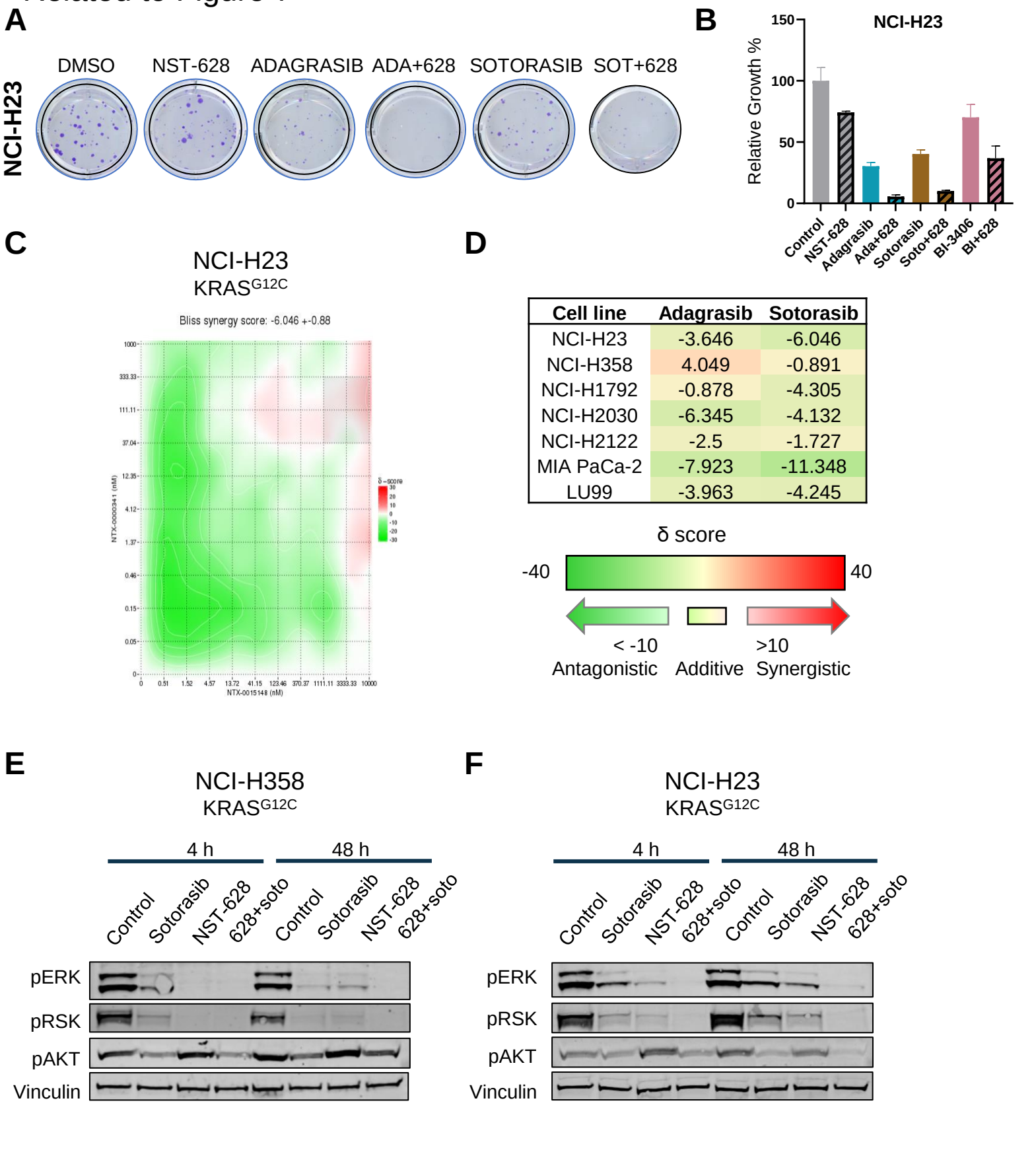
Related to Figure 6



Supplementary Figure S11. (A) NST-628 blood plasma levels in mice treated with 0.3, 1, or 3 mg/kg NST-628 QD for 7 days. Samples were collected and analyzed by LC/MS/MS. (B) Human oral PK profiles are simulated based on predicted PK modeling parameters and assuming F% = 50% and tumor stasis concentration (TSC) is based on pERK readouts from the HCT116 model in Figure 5 (C) Mouse body weights and (D) individual tumor volumes of SK-MEL-2-luc tumors from Figure 6 treated with treated with 0.3 mg/kg QD trametinib, 0.3 mg/kg QD avutometinib, 3 mg/kg QD or 1.5 mg/kg BID NST-628 (E) Mouse body weights and (F) individual tumor volumes of MeWo-luc tumors from Figure 6 treated with 25 mg/kg QD tovorafenib or 0.3, 1, 3 mg/kg QD NST-628.

Supplementary Figure S12

Related to Figure 7



Supplementary Figure S12. (A) NCI-H23 cells treated with 10 nM NST-628, 100 nM adagrasib, 100 nM sotorasib, or a combination of NST-628 with adagrasib or sotorasib for 14 days and stained with crystal violet (B) Quantification of assay in A. (C) Bliss Synergy score plot of the combination of a dose response of NST-628 combined with a dose response of sotorasib read out by CTG after 72 h of treatment in the NCI-H23 cell line. (D) Summary of Bliss Synergy scores across $KRAS^{G12C}$ mutant models treated with dose response combinations of NST-628 with sotorasib or adagrasib treated as in C. (E) NCI-H358 of (F) NCI-H23 treated with 20 nM NST-628, 100 nM sotorasib, or combination for 4 and 48 h and blot analysis for phospho-ERK, phospho-RSK, phospho-AKT, and vinculin as a loading control