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## Comparative Analysis and Isoform-Specific Therapeutic Vulnerabilities of KRAS Mutations in Non-Small Cell Lung Cancer

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1 **Comparative analysis and isoform-specific therapeutic vulnerabilities of KRAS**  
2 **mutations in non-small cell lung cancer**

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33

34 **Running title:** distinct bio-pathological features of KRAS mutants in NSCLC

35

36 **Conflicts of interest**

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13 NextCure, personal fees from Novartis, personal fees from EMD Serono, personal fees  
14 from Panvaxal/NovaRx. J.K. has received consultant fees from and served on advisory  
15 boards for Boehringer-Ingelheim. A.J.R. has received personal fees from Boehringer-  
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18 The other authors declare no competing interests.

19

## 20 **Translational relevance**

21 *KRAS* is the most commonly mutated driver oncogene in solid tumors, including non-small  
22 cell lung cancer (NSCLC), and *KRAS* mutations have traditionally been associated with  
23 poor prognosis and treatment resistance. Comprehensive genomic, biologic and  
24 clinicopathologic analysis of *KRAS* mutant variants in pre-clinical models and samples  
25 from patients with non-squamous NSCLC revealed isoform-dependent therapeutic  
26 vulnerabilities of these tumors. Our results suggest that the use of specific genetically  
27 defined *in vitro* tools together with the development of large patient-derived datasets  
28 facilitates the identification of potentially actionable vulnerabilities for *KRAS* mutant  
29 NSCLC.

30

## 31 **Abstract**

32 **Purpose:** activating missense mutations of *KRAS* are the most frequent oncogenic  
33 driver events in lung adenocarcinoma (LUAD). However, *KRAS* isoforms are highly

1 heterogeneous, and data on the potential isoform-dependent therapeutic vulnerabilities  
2 are still lacking.

3 **Experimental design:** we developed an isogenic cell-based platform to compare  
4 the oncogenic properties and specific therapeutic actionability of KRAS mutant isoforms.  
5 In parallel, we analyzed clinicopathologic and genomic data from 3560 patients with non-  
6 small cell lung cancer (NSCLC) to survey allele-specific features associated with  
7 oncogenic KRAS mutations.

8 **Results:** In isogenic cell lines expressing different mutant KRAS isoforms, we  
9 identified isoform-specific biochemical, biological and oncogenic properties both *in vitro*  
10 and *in vivo*. These exclusive features correlated with different therapeutic responses to  
11 MEK inhibitors, with *KRAS* G12C and Q61H mutants being more sensitive compared to  
12 other isoforms. *In vivo*, combined *KRAS* G12C and MEK inhibition was more effective  
13 than either drug alone. Among patients with NSCLCs which underwent comprehensive  
14 tumor genomic profiling, *STK11* and *ATM* mutations were significantly enriched among  
15 tumors harboring *KRAS* G12C, G12A, and G12V mutations. *KEAP1* mutation was  
16 significantly enriched among *KRAS* G12C and *KRAS* G13X LUADs. *KRAS* G13X mutated  
17 tumors had the highest frequency of concurrent *STK11* and *KEAP1* mutations.  
18 Transcriptomic profiling revealed unique patterns of gene expression in each *KRAS*  
19 isoform, compared to *KRAS* wild-type tumors.

20 **Conclusions:** This study demonstrates that *KRAS* isoforms are highly  
21 heterogeneous in terms of concurrent genomic alterations and gene expression profiles,  
22 and that stratification based on *KRAS* alleles should be considered in the design of future  
23 clinical trials.

24

## 25 **Introduction**

26 *KRAS* is one of the most commonly mutated oncogenes in human cancer, with  
27 selectively high frequency in tumors of the pancreas, colon, and lung. In non-small cell  
28 lung cancer (NSCLC), *KRAS* mutations are detected in about 30% of patients and are  
29 associated with poor prognosis (1,2). Several strategies have been investigated over the  
30 last decades to target *KRAS* mutation in lung cancer, including the inhibition of  
31 downstream effectors such as MEK (3,4). However, none of these strategies was effective  
32 in clinical trials, and the optimal first-line therapy for advanced *KRAS*-mutant lung  
33 adenocarcinoma (LUAD) still consists of PD-(L)1 blockade alone or in combination with  
34 platinum-based chemotherapy (5). More recently, alternative strategies targeting *KRAS*-



1 mutant lung cancer have been investigated in preclinical models, and are in various  
2 stages of clinical development (1,6,7).

3 To date, the greatest success in targeting *KRAS* mutations is represented by the  
4 development of isoform-specific direct *KRAS* inhibitors. Recent data from early phase  
5 clinical trials of *KRAS* G12C direct inhibitors sotorasib and adagrasib have shown  
6 encouraging activity in *KRAS* G12C-mutant NSCLCs, with responses occurring in ~30-  
7 40% of patients (8–10). Based on the results of the CodeBreak 100 study and KRYSTAL-  
8 1, sotorasib and adagrasib were granted accelerated FDA approval and breakthrough  
9 therapy designation (BTD), respectively, for patients with NSCLC who have received on  
10 prior systemic therapy. Nevertheless, an urgent need remains for better stratification and  
11 effective therapeutic strategies to improve outcomes for *KRAS*-mutant cancer patients.

12 Although RAS mutations were discovered over 30 years ago (11), the detailed  
13 understanding of the biological properties of oncogenic *KRAS* is still far to be complete.  
14 New aspects of *KRAS* biology have been recently described, including the requirement of  
15 dimerization for oncogenic signaling (12), lipid-sorting specificity into the membrane (13),  
16 tissue-specific co-mutation networks (14).

17 One intriguing aspect of *KRAS* biology is the potential impact of distinct *KRAS*  
18 activating mutations on downstream signaling and drug sensitivity (15,16). Until very  
19 recently, RAS proteins with alterations in codon 12, 13, or 61 have been considered  
20 oncogenic equivalents; however, emerging data suggest functional differences for each  
21 RAS mutation in colon cancer (17), melanoma (18) and NSCLC, where different mutant  
22 *KRAS* proteins are known to be heterogeneous in terms of clinical outcome (19–20).  
23 Recently studies in isogenic cell lines demonstrated that each individual mutation  
24 generates a distinct oncogenic and network response (14,15,21–23). In terms of  
25 translational impact, the study of differences among specific *KRAS* mutants by means of  
26 more informative experimental approaches will be fundamental to discover new  
27 therapeutic strategies for individual *KRAS* mutations.

28 Here, we developed an isogenic system where a series of mutant *KRAS* isoforms  
29 commonly found in lung cancer were directly compared to highlight distinct GTP-loading  
30 levels, differential drug sensitivity to MEK inhibitors and unique growth properties *in vivo*.  
31 To gather evidence on whether these differences also translated in different clinical  
32 outcomes we investigated the impact of the different *KRAS* isoforms on survival in a large  
33 cohort of patients with non-squamous NSCLC. Together, these results suggest that  
34 genomic co-alterations and residue-specific properties of *KRAS* mutations have important

1 biologic and clinical implications.

2

### 3 **Methods**

#### 4 *Generation of KRas<sup>lox</sup> KRAS<sup>MUT</sup> cells*

5 KRas<sup>lox</sup> KRAS<sup>MUT</sup> cells were generated as previously described (12). Briefly, KRAS<sup>MUT</sup>  
6 retroviral constructs were created by point mutagenesis from pBABE HA-tagged KRAS<sup>WT</sup>  
7 plasmid (a gift from Channing Der, Addgene plasmid #75282). Retroviruses were  
8 generated by co-transfection of pBABE plasmids together with pAmpho plasmid into 293T  
9 cells using FuGENE® HD Transfection Reagent (Promega). The retroviruses were  
10 transduced into *HRas*<sup>-/-</sup>; *NRas*<sup>-/-</sup>; *KRas*<sup>lox/lox</sup> MEFs (24) followed by puromycin selection (1  
11 µg/ml). Selected cells were then cultured in the presence of 4-hydroxytamoxifen (4OHT)  
12 (Sigma, 600 nM) for at least 10 days in order to achieve complete deletion of endogenous  
13 *KRas* alleles. Cells were not cultured longer than 3 months after thawing from frozen  
14 stocks and were routinely checked for *Mycoplasma* as determined by the Mycoplasma  
15 Plus PCR Primer Set (Agilent).

16

#### 17 *Growth assays in vitro*

18 Cells (1x10<sup>3</sup>) were seeded in 96-well plates in 150 µl complete medium. The following  
19 day, cells were PBS-washed and 150 µl of medium with different concentrations of FBS  
20 was added to the plates. Cells were then incubated in the IncuCyte Zoom for real-time  
21 imaging, with three fields imaged per well under 10x magnification every two hours for a  
22 total of 60 to 96 hours. Data were analyzed using the IncuCyte Confluence version 1.5  
23 software, which quantified cell surface area coverage as confluence values. IncuCyte  
24 experiments were performed in triplicate. A single representative growth curve is shown  
25 for each condition. For hypoxic conditions, cells (1x10<sup>3</sup>) were seeded in 96-well plates in  
26 150 µl complete medium. The following day, cells were PBS-washed and 150 µl of  
27 medium with different concentrations of FBS was added to the plates that were incubated  
28 in 1% O<sub>2</sub> incubator. Cell viability was assessed by CellTiter-Glo® 2.0 (Promega) at the  
29 indicated time points.

30

#### 31 *Analysis of MEK/ERK inhibitor sensitivities*

32 Data from a large-scale pharmacogenomics study, the Cancer Genome Project (CGP,  
33 dataset version 2020), were accessible from <http://www.cancerrxgene.org>. 310 lung,  
34 pancreas and colon cancer cell lines and their drug sensitivity values to MEK/ERK

1 inhibitors in the CGP dataset were used for analysis. In these data, the natural logarithm  
2 of the IC<sub>50</sub> value represents the drug sensitivity value. Cell lines were grouped according  
3 to KRAS mutational subtypes and pairwise comparisons were performed using the Mann-  
4 Whitney U test.

5

#### 6 *Drug-response assays*

7 Cells ( $1 \times 10^3$ ) were seeded in 96-well plates. The following day, cells were treated with  
8 drugs using a ten-point dose titration scheme. After 72 hours, cell viability was assessed  
9 using the colorimetric MTS assay. Absolute inhibitory concentration (IC) values were  
10 calculated using four-parameter logistic curve fitting. All experimental points were a result  
11 of three to six replicates, and all experiments were repeated at least three times. The data  
12 was graphically displayed using GraphPad Prism 5 for Windows (GraphPad Software).  
13 Each point (mean  $\pm$  standard deviation) represents growth of treated cells compared to  
14 untreated cells. The curves were fitted using a non-linear regression model with a  
15 sigmoidal dose response. For synergy distribution assays, cells were seeded at 1500 cells  
16 per well in a 384-well plate (Corning). The following day, cells were treated with  
17 selumetinib and SHP099, varying concentration from 0.25  $\mu$ M to 15  $\mu$ M and from 0.5  $\mu$ M  
18 to 15  $\mu$ M, respectively. After 72 hours, cell viability was measured by CellTiter-Glo 2D  
19 (Promega). Four replicates of each mutant were analyzed implementing LOEWE model  
20 (Combenefit software); experiments were repeated three times.

21

#### 22 *Ras-GTP Pull-down*

23 Cell were grown in 0.1% FBS for 24 hours, stimulated with EFG (Thermo Fisher  
24 Cat#PHG0311) 50ng/ml for 5 minutes and Ras-GTP levels were assessed by Active Ras  
25 Detection Kit (Cell Signaling, Cat#8821) using Raf-RBD fused to GST to bind active (GTP-  
26 bound) Ras. Protein lysates (500  $\mu$ g) were incubated with 30  $\mu$ l glutathione resin and GST  
27 protein binding domains for one hour at 4°C to capture active small GTPases according to  
28 the manufacturer's protocol. After washing, the bound GTPase was recovered by eluting  
29 the GST-fusion protein from the glutathione resin. The purified GTPase was detected by  
30 Western blot using mouse monoclonal anti-KRAS (F234) (Santa Cruz Biotech, Cat#sc-  
31 30).

32

#### 33 *3D organoid culture and growth assay*

1 After trypsinization, cells from the plastic culture were resuspended in ice-cold Matrigel  
2 (Corning) and incubated on pre-warmed 6 cm plate to solidify. Organoids were cultured in  
3 Renaissance Essential Tumor Medium (RETM; Cellaria) with B-27 supplement (Thermo  
4 Fisher Scientific), and passaged over three times before performing any experiments. For  
5 the growth assay, organoids in Matrigel were dissociated into single cells using TrypLE  
6 Express (Invitrogen) at 37°C. A thousand of cells were plated into each well of 384-well  
7 ultra-low attachment microplates (Corning) with the media and 10% Matrigel. On the next  
8 day, cells were treated with dose titration of trametinib, or selumetinib for three days (from  
9 1 nM to 10  $\mu$ M). Each dose had more than three replicates. The viability assay was tested  
10 using CellTiter-Glo 3D (Promega) according to the manufacturer's protocol. The statistical  
11 significance was accessed using ANOVA and Turkey's post-test for multiple comparison.

12

### 13 *In vivo assays*

14 Crl:NU-Foxn1<sup>nu</sup> mice (females, 8-week-old) were purchased from Charles River. For lung  
15 colonization assays, KRas<sup>lox</sup> KRAS<sup>MUT</sup> cells ( $1 \times 10^6$ ) were injected tail vein as single-cell  
16 suspension in 200  $\mu$ l of sterile PBS. Mice were sacrificed synchronously at a single time  
17 point of one month and lungs collected in formalin. For drug treatment assays, KRas<sup>lox</sup>  
18 KRAS<sup>MUT</sup> cells ( $2 \times 10^6$ ) were injected subcutaneously in a 1:1 mix of serum-free DMEM  
19 and Matrigel (phenol red-free; BD Biosciences) in both flanks of recipient mice. Once a  
20 palpable tumor formed, were randomly assigned to either selumetinib, sotorasib or vehicle  
21 treatment and measurements were taken daily using calipers. Selumetinib and sotorasib,  
22 resuspended in 2% Hydroxypropyl Methyl Cellulose (HPMC), 1% Tween 80 were  
23 administered daily by oral gavage at a dose of 50 mg/kg. For IVIS longitudinal monitoring,  
24 KRas<sup>lox</sup> KRAS<sup>MUT</sup> cells were transduced with pFUGW-Pol2-ffLuc2-eGFP (Addgene  
25 plasmid #71394) and FACS-sorted for GFP expression. KRas<sup>lox</sup> KRAS<sup>MUT</sup>-luciferase cells  
26 ( $2 \times 10^6$ ) were injected in the tail vein of 8-weeks old NCr nude mice (Taconic Biosciences,  
27 NY) as single-cell suspension in 200  $\mu$ l of sterile PBS and were monitored using an IVIS  
28 imaging system (Perkin Elmer, MA). All care and treatment of experimental animals were  
29 in strict accordance with Good Animal Practice as defined by the US Office of Laboratory  
30 Animal Welfare and approved by the Dana-Farber Cancer Institute Institutional Animal  
31 Care and Use Committee and by the Italian Health Minister (authorization n° 1227/2020-  
32 PR).

33

### 34 *Study Population*

1 Clinicopathologic and genomic data from patients with NSCLC whose tumors underwent  
2 targeted next generation sequencing at the Dana-Farber Cancer Institute who had  
3 provided written informed consent to institutional review board-approved protocols  
4 DF/HCC#02-180, #11-104, #13-364, and/or #17-000 were included in this study. This  
5 study was conducted in agreement to the Declaration of Helsinki.

6

#### 7 *Tumor mutational burden assessment and PD-L1 assessment*

8 Tumor mutational burden (TMB), defined as the number of somatic, coding, base  
9 substitution and indel mutations per megabase (Mb) of genome examined was calculated  
10 from the DFCI OncoPanel next generation sequencing (NGS) platform. PD-L1 expression  
11 on tissue was assessed by immunohistochemistry using an anti-PD-L1 rabbit monoclonal  
12 antibody (clone E1L3N, Cell Signaling Technology).

13

#### 14 *Clinical outcomes*

15 Overall survival (OS) was defined as the time from the date of initial diagnosis to death.  
16 Patients who were still alive at the data cut-off were censored at the date of last contact.  
17 Overall survival was compared between KRAS mutated and KRAS wild type cases, as  
18 well as across KRAS isoforms.

19

#### 20 *Co-occurrence and mutual exclusivity analysis*

21 Fisher's exact test p-values and conditional odds ratios were used to assess co-  
22 occurrence and mutual exclusivity for genes with at least 2% frequency in the relevant  
23 groups. Positive odds ratios represented tendency to co-occurrence and negative odds  
24 ratios represented tendency to mutually exclusivity. Multiple comparison correction was  
25 performed using the Benjamini-Hochberg procedure using the qvalue package in R.

26

#### 27 *Gene expression analysis from the TCGA*

28 Gene expression data were downloaded from the Firehose website (TCGA Firehose  
29 Legacy version) while somatic mutation data were downloaded from cBioPortal website  
30 (cbioportal.org). The RSEM V2 values were used to represent gene expression and genes  
31 with counts less than 10 were filtered out. Gene expression profiles were analyzed  
32 according to KRAS mutation status. Median expression within each group was used to  
33 estimate expression fold-change (FC) to minimize the possible impact of outlier samples.  
34 Gene differential expression analyses across KRAS allele subgroups were conducted

1 using R package DESeq2. P-values were corrected for multiple hypothesis testing via  
2 false discovery rate (FDR) adjustment. Fold-change threshold of an absolute value greater  
3 than 1.5 and FDR adjusted P-value threshold less than 0.1 were utilized to identify  
4 differentially expressed genes. Pathway enrichment analyses were conducted separately  
5 for up- and down-regulated genes using R package Reactome.

6

### 7 *Statistical analysis*

8 We summarized continuous and categorical variables using percentages and medians.  
9 To test for examine differences between continuous variables we used the Wilcoxon-Rank  
10 Sum test and Kruskal-Wallis, when appropriate. The Fisher's exact test was used to  
11 compare associations between categorical variables. Estimate event-time distributions  
12 were examined using the Kaplan-Meier methodology, and the log-rank tests was used to  
13 test for differences in event-time distributions. Hazard ratios were estimated using the Cox  
14 regression models, as previously described (25). To investigate MEK/ERK inhibitor  
15 sensitivities for different KRAS mutant isoforms across cancers, we grouped cancer cell  
16 lines based on their KRAS mutation status into the different groups. Mann-Whitney U test  
17 was used to compare drug sensitivities between two groups. All P values were two-sided  
18 with confidence intervals set at the 95% level.  $P < 0.05$  were defined as significant.  
19 Multiple comparison correction was performed using the Benjamini-Hochberg procedure  
20 using the qvalue package in R. All statistical analyses were performed using R version  
21 3.6.3.

22

### 23 **Data availability statement**

24 The Institutional clinicopathologic and genomic data that support the finding of our study  
25 are available upon reasonable request from the corresponding author. RNAseq data used  
26 in this study are publicly available from the TCGA (accession number: phs000178).  
27 Genomic data from the AACR project GENIE (Genomics Evidence Neoplasia Information  
28 Exchange) are publicly available (phs001337).

29

## 30 **Results**

### 31 **Generation of KRas<sup>lox</sup>KRAS<sup>MUT</sup> cell lines panel**

32 Among *KRAS*-mutant lung cancer, codon 12 mutations predominate, accounting for  
33 nearly 90% of all *KRAS* mutations, followed by mutations in codons 13 and 61 (14). One  
34 question that arises from somatic genetic analysis is whether different *KRAS* mutations

1 determine the clinical aspects of a given cancer. To investigate the role of individual KRAS  
2 isoforms on the biology of mutant *KRAS* avoiding context-dependent differences  
3 associated with different genetic backgrounds, we developed an isogenic system  
4 generated from *HRas*<sup>-/-</sup>; *NRas*<sup>-/-</sup>; *KRAS*<sup>lox/lox</sup> mouse embryonic fibroblasts (MEFs) (24). In  
5 these cells, *KRAS*<sup>lox/lox</sup> alleles excision is controlled by a resident 4-hydroxytamoxifen  
6 (4OHT)-dependent CRE<sup>ERT2</sup> recombinase. We transduced *HRas*<sup>-/-</sup>; *NRas*<sup>-/-</sup>; *KRAS*<sup>lox/lox</sup>  
7 MEFs with retroviruses encoding for different human HA-tagged *KRAS* mutants, including  
8 the most frequent mutant isoforms detected in human lung adenocarcinoma (G12C,  
9 G12D, G12V, G12A, G13D, Q61H) (12).

10 In all cell lines, the presence of the respective *KRAS* mutation was confirmed by  
11 Sanger sequencing (data not shown). Cells were treated with 4OHT to abolish the  
12 expression of endogenous wild-type KRas, thus allowing the characterization of specific  
13 oncogenic *KRAS* mutations in isogenic cell lines without the interference of H- or NRas  
14 isoforms or the endogenous wild-type KRas (herein referred to KRas<sup>lox</sup> KRAS<sup>MUT</sup> system)  
15 (**Figure 1A**). Interestingly, all *KRAS* mutants showed increased expression upon deletion  
16 of the endogenous wild-type KRas allele. Therefore, we utilized only 4OHT-treated cells in  
17 order to eliminate any bias imposed by the wild-type allele. In full-serum condition,  
18 phosphorylation of ERK, MEK, SRC and AKT was slightly increased in all *KRAS* mutants  
19 compared to wild-type control cells (**Figure 1B**). In contrast, STAT3 and EGFR showed  
20 marked differences between mutants in terms of both phosphorylation and expression  
21 levels (**Figure 1B**). Remarkably, KRAS<sup>GTP</sup> levels upon stimulation with EGF showed  
22 profound variability among mutants, with *KRAS* G12D and G12V being already saturated  
23 in untreated condition in contrast with the other mutants which are still responsive to  
24 mitogenic stimulation (**Figure 1C**).

## 25 26 **Growth properties of different *KRAS* mutants *in vitro* and *in vivo***

27 Next, we aimed at characterizing the proliferation kinetics of KRas<sup>lox</sup> KRAS<sup>MUT</sup> cells  
28 both *in vitro* and *in vivo*. We tested growth properties of different *KRAS* mutants by  
29 Incucyte in full-serum and in starving conditions. All mutants displayed a strong  
30 proliferative advantage over wild-type *KRAS* controls in full-serum, nevertheless no  
31 significant differences were observed among mutants (**Figure 1D**). Under starving  
32 conditions however, a clear stratification was evident, with G12D, and G12V to a lesser  
33 extent, showing the best fitness over the other *KRAS* mutants (**Figure 1D**). Of note, G12D  
34 and G12V are the mutant isoforms displaying higher GTP-bound fraction in limited serum

1 conditions (**Figure 1C**). Similar results were obtained in hypoxic conditions, with G12D  
2 mutant cells showing improved fitness compared to the other *KRAS* mutants upon  
3 concomitant starvation (**Figure S1A**). In 3D conditions, all *KRAS* mutant isoforms showed  
4 comparable growth kinetics with the exception of Q61H which was significantly less  
5 proliferative (**Figure 1D**). The advantage of G12D versus the other mutants was  
6 maintained also in tumor growth assays *in vivo*. We injected  $KRAs^{lox} KRAS^{MUT}$  cells  
7 expressing different mutants into the tail vein of nude mice and euthanized the animals  
8 after one month in order to check for colonization property into the lungs. At histological  
9 evaluation, G12D mutant cells displayed tumor nodules formation into the lungs, whereas  
10 all the other mutants did not (**Figure 1E**). Using the same mutant cells expressing  
11 luciferase gene, we noninvasively monitored tumor growth in the mice. As consistent to  
12 Figure 1E and *in vitro* results, G12D and G12V mutants were established in the mouse  
13 lung much quicker than other mutants (**Figure S1B-C**). These results confirmed an *in vivo*  
14 advantage for  $KRAS^{G12D}$  mutants (26), although further assessment at longer time points  
15 should be performed in order to detect delayed tumor onset in mutants other than G12D.

16

### 17 **Sensitivity to MEK inhibitors and G12C inhibitors *in vitro* and *in vivo***

18 Due to the prominent role of the MAPK pathway in *KRAS* signaling, MEK inhibitors were  
19 thought to be able to achieve disease control in *KRAS* mutant tumors. However, phase III  
20 clinical trials demonstrated no overall benefit compared to chemotherapy (4).  
21 Retrospective analysis in different sub-populations of *KRAS* codon mutations showed that  
22 patients with *KRAS* G12C or G12V receiving selumetinib+docetaxel had improved overall  
23 survival compared with other *KRAS* mutations, suggesting that the type of *KRAS*  
24 mutations may impact on MEK inhibitors sensitivity in LUAD (20). We next surveyed the  
25 CGP pharmacogenomic dataset (27) to identify correlations between sensitivity to MEK  
26 inhibitors and *KRAS* mutant isoforms in a broad panel of lung, pancreas and colon cancer  
27 cell lines. We could not observe any obvious trend for increased sensitivity for specific  
28 isoforms other than G12R, likely due to the limited representation of small number of cell  
29 lines (**Figure S2**). Considering that cell lines heterogeneity poses challenging limits to the  
30 characterization of *KRAS* isoform as single variable to predict MEK inhibitors sensitivity,  
31 we tested a panel of MAPK inhibitors in our isogenic  $KRAs^{lox} KRAS^{MUT}$  system.  
32 GSK1120212 (trametinib) showed similar antiproliferative effects in all *KRAS* mutants,  
33 whereas selumetinib and the other MAPK inhibitors exhibited up to 10-fold IC50 variability  
34 in different *KRAS* isoforms, being G12C and Q61H among the most sensitive (**Figure 2A**



1 and **Figure S3**) (28,29). The same trend was observed in 3D culture (**Figure 2B**).  
2 Western blot assessment of the MAPK pathway confirmed dose-dependent reduction of  
3 pERK in selumetinib-treated KRas<sup>lox</sup> KRAS<sup>MUT</sup> cells, with the exception of G12A and  
4 G13D mutants (**Figure 2C**). To validate these findings into an *in vivo* setting, we implanted  
5 KRas<sup>lox</sup> KRAS<sup>MUT</sup> cells into nude mice and followed tumor growth in presence or absence  
6 of selumetinib treatment (50mg/kg daily). In agreement with our findings in 2D and 3D  
7 conditions, we could stratify the outcome as tumor regression (G12C and Q61H), stable  
8 disease (G12D, G12V and G13D) and no response (G12A) based on tumor volume fold  
9 change on treatment (**Figure 2D**).

10 Considering that pre-clinical *in vivo* studies demonstrated that in *KRAS* mutant  
11 LUAD, combined SHP2/MEK inhibition is therapeutically effective (30,31), we tested the  
12 same combination in KRas<sup>lox</sup> KRAS<sup>MUT</sup> cells. Interestingly, we observed that combined  
13 SHP2/MEK blockade was more effective in G12C, G12D and G12V mutants compared to  
14 the other mutants, in agreement with previous findings (31,32) (**Figure S4**).

15 Given the very recent FDA approval of the selective G12C inhibitor sotorasib,  
16 (CodeBreak 100, NCT03600883), we aimed at assessing the efficacy of G12C inhibition  
17 in our isoform-specific KRAS<sup>G12C</sup> system, both *in vitro* and *in vivo*. As expected, drug-  
18 response curves with sotorasib demonstrated a clear selectivity for G12C mutant cells  
19 compared to the other *KRAS* mutant isoforms (**Figure 3A**). To determine how G12C  
20 inhibition compared with MEK inhibition in this model, we next implanted KRas<sup>lox</sup>  
21 KRAS<sup>G12C</sup> cells into nude mice and randomized these mice to receive either vehicle,  
22 selumetinib (50mg/kg daily), sotorasib (50mg/kg daily), or selumetinib + sotorasib.  
23 Interestingly, selumetinib exerted better therapeutic response in comparison to sotorasib,  
24 possibly likely due to the delay in implementing feed-back mechanisms for MEK inhibitors  
25 as compared to selective G12C inhibitors as single agent. Importantly, combined  
26 treatment with selumetinib + sotorasib resulted in complete growth abrogation and  
27 disease control in mice treated with this combination (**Figure 3B-E**). This preliminary  
28 finding further supports the hypothesis that combined *KRAS* G12C and MEK inhibition  
29 may improve clinical outcomes in patients with *KRAS* mutant NSCLC over G12C inhibition  
30 alone. A phase Ib clinical trial of sotorasib in combination with other anticancer agents,  
31 including MEK inhibitors is currently ongoing (NCT04185883).

32  
33 **Clinicopathological and genomic correlation of *KRAS* variants in patients with non-**  
34 **squamous NSCLC**

1 In order to translate our findings based on genetically defined cell lines to a  
2 clinically relevant context, a total of 3560 patients with NSCLCs which underwent  
3 successful comprehensive tumor genomic profiling at the Dana-Farber Cancer Institute  
4 were identified. Of these, 3126 (87.8%) were non-squamous NSCLC and were included in  
5 the final analysis (**Figure S5A**). The median age of the 3126 patients with non-squamous  
6 NSCLC was 66 (range: 18-99), 61.2% were female, 18.6% were current smokers, 58.4%  
7 were former smokers, and 18.6% were never smokers. *KRAS* mutation (*KRAS*<sup>MUT</sup>)  
8 occurred in 1131 cases (36.2%), while the remaining 1995 cases (63.8%) had a *KRAS*  
9 wild type (*KRAS*<sup>WT</sup>) genotype (**Table S1**). The most common *KRAS* mutations identified  
10 occurred at codon 12 (990/1131, 87.5%), of which the *KRAS* G12C was the most frequent  
11 (476/990, 47.2%) (**Figure S5B**).

12 We first investigated differences in clinicopathologic and genomic factors according  
13 to *KRAS* mutation status. Compared to *KRAS*<sup>WT</sup> cases, patients with *KRAS*<sup>MUT</sup> tumors  
14 were more likely to be older ( $P<0.001$ ), females ( $P<0.0001$ ), current/former smokers  
15 ( $P<0.0001$ ), and to have adenocarcinoma histology ( $P<0.01$ ), consistently with previous  
16 reports (33,34). Baseline clinicopathologic features of patients with *KRAS*<sup>WT</sup> versus  
17 *KRAS*<sup>MUT</sup> are shown in **Table S2**. We next focused on co-mutations in preselected genes  
18 of interest which define major subsets of NSCLC, including *TP53*, *STK11*, and *KEAP1*. Of  
19 note, while mutations in *TP53* were more likely to occur in *KRAS*<sup>WT</sup> tumors ( $P<0.0001$ ),  
20 *STK11* and *KEAP1* were significantly enriched in *KRAS* mutated tumors ( $P<0.0001$ )  
21 (**Figure S5C**). We also examined whether PD-L1 expression and TMB distributions  
22 differed between *KRAS*<sup>MUT</sup> and *KRAS*<sup>WT</sup> Nsq-NSCLCs. We noted that both median PD-L1  
23 expression (10 vs 5%,  $P<0.001$ ) and median TMB (9.8 vs 8.4 mut/Mb,  $P<0.0001$ ) were  
24 significantly higher in *KRAS*<sup>MUT</sup> versus *KRAS*<sup>WT</sup> tumors (**Figure S5D**).

25 Next, we examined the impact of *KRAS* mutation on survival among patients with Nsq-  
26 NSCLC. While we found no significant impact of *KRAS* mutation on OS in all comers (HR:  
27 1.07,  $P=0.21$ ), as well as among patients with stage I-III Nsq-NSCLC (HR: 1.08,  $P=0.39$ ),  
28 we observed a significant deleterious impact of *KRAS* mutation among patients with stage  
29 IV tumors (16.2 versus 25.5 months, HR:1.37,  $P<0.0001$ ) (**Figure 4A-C**).

30 Although we identified a detrimental impact of *KRAS* mutation among patients with  
31 advanced Nsq-NSCLC, this difference likely reflects the use of highly effective targeted  
32 therapies among patients with *KRAS* wild type tumors harboring *EGFR* mutations and  
33 *ALK* fusions.

1           Because we have shown that different *KRAS* mutation have different *in vitro* and *in*  
2 *vivo* growth properties, and differential responses to MEK inhibitors, we lastly investigated  
3 whether different *KRAS* mutation impacted survival in patients with Nsq-NSCLC. The  
4 clinicopathologic characteristics of patients according to *KRAS* alleles are shown in the  
5 **Table S3**. Consistently with previous reports (35) we found a significantly higher  
6 proportion of never smokers among patients with *KRAS* G12D mutation (21.8%) as  
7 opposed to the other *KRAS* variants (<10%). While there was no difference in terms of  
8 enrichment in *TP53* mutation across the *KRAS* variants, the rates of concurrent *STK11*  
9 and *KEAP1* mutations were highest among *KRAS* codon 13 mutations (**Figure S5C**).  
10 Pairwise comparisons between *KRAS* alleles in terms of *TP53*, *STK11*, and *KEAP1* co-  
11 mutation patterns are shown in **Figure S6A**. When we examined PD-L1 expression and  
12 TMB distributions in this across the different *KRAS* alleles, we found that the median TMB  
13 differed significantly across the seven groups, with *KRAS* G12D mutated tumors having  
14 the lowest number of non-synonymous mutations/megabase (**Figure S5D**). By contrast,  
15 no difference in PD-L1 expression was observed among these groups (**Figure S5D**).  
16 Pairwise comparisons between *KRAS* alleles in terms of PD-L1 and TMB distributions are  
17 shown in **Figure S6B**.

18           We lastly analyzed the impact of *KRAS* alleles on survival in patients with non-  
19 squamous NSCLC. We found no significant difference across *KRAS* mutation subtype in  
20 all comers, as well as among those with stage I-III or stage IV disease (**Figure 4D-F**).

### 21 22 ***KRAS* variants have different co-mutation patterns and gene-expression profiles in** 23 **patients with non-small cell lung cancer**

24           Although we did not find differences in survival in patients with NSCLC with  
25 different *KRAS* alleles, we identified several differences in clinicopathologic and genomic  
26 factors among NSCLC harboring different *KRAS* alleles in the DFCI cohort. Therefore, we  
27 hypothesized that one of the reasons by which our findings in isogenic models did not  
28 translate into different clinical outcomes, could be the biological heterogeneity of *KRAS*-  
29 mutant LUADs. To further dissect this heterogeneity in patients with *KRAS* mutant LUADs,  
30 we interrogated a large publicly available cohort of 12,931 LUADs (GENIE v.9.0) which  
31 underwent tumor genomic profiling to comprehensively determine differences in co-  
32 mutation patterns across *KRAS* variants. After QC removing duplicate samples and gene  
33 annotation, 10,663 unique samples were included in the final analysis. *KRAS* mutation  
34 was detected in 3,843 of cases (36%). In this cohort, the most common genomic

1 alterations included *TP53* (33%), *STK11* (16%), *ATM* (11%), *KEAP1* (11%), and *RBM10*  
2 (7%) (**Figure 5A**). When compared to *KRAS* wild-type cases, however, *KRAS* mutant  
3 LUADs were found to be significantly enriched in *STK11*, *KEAP1*, *ATM*, *RBM10*  
4 mutations, while *TP53*, *EGFR*, *BRAF*, *ERBB2* were less likely to co-occur with any *KRAS*  
5 mutation (**Figure S7A-B**).

6 We next focused on the individual *KRAS* variants and compared the co-mutation  
7 patterns of each *KRAS* variant with *KRAS* wild-type LUADs, as reference. We identified  
8 that all *KRAS* variant tended to be mutually exclusive with *EGFR* mutation, and were more  
9 likely to lack concurrent *TP53* mutations (**Figure 5B**). Of note, among LUADs with codon  
10 12 mutations, *STK11* and *ATM* mutations was significantly enriched among tumors  
11 harboring *KRAS* G12C, G12A, and G12V mutations, while such enrichment was not  
12 detected in LUADs with *KRAS* G12D (**Figure 5B**). Additionally, we also found that *KEAP1*  
13 mutation was significantly enriched among *KRAS* G12C and *KRAS* G13X LUADs.  
14 Importantly, the highest frequency of concurrent *STK11* and *KEAP1* mutation was  
15 observed in tumors with *KRAS* codon 13 mutations (**Figure S7C**). Pairwise comparisons  
16 in concurrent and mutually exclusive mutations between each *KRAS* variant and the  
17 remaining *KRAS* alleles are reported in **Figure S8A-B**.

18 Having shown that the *KRAS* mutant LUADs are highly heterogeneous in terms of  
19 concurrent genomic alterations, we lastly examined whether tumors with different *KRAS*  
20 mutations had also different gene expression profiles. To address this question, we  
21 performed a gene ontology analysis of differentially expressed genes in each *KRAS*  
22 variant versus *KRAS* wild-type tumors in the TCGA LUAD cohort. We found that several  
23 pathways were uniquely up- and downregulated in specific *KRAS* isoform (**Figure 5C-D**).  
24 Interestingly, *KRAS* G12D mutant LUADs showed a significant downregulation of  
25 pathways involved in chromosome maintenance, DNA double break repair, and cellular  
26 senescence (**Figure 5C**), while *KRAS* G13X LUADs had a significant downregulation of  
27 pathways involved in innate and adaptive immunity, including PD-1, CD28, INF $\gamma$ , and IL-  
28 2 signaling, which is consistent with the high rate of concurrent *STK11* and/or *KEAP1*  
29 (36–37) mutations we identified in this group of LUADs. A full list of differentially regulated  
30 pathways in all *KRAS* isoforms is reported in **Table S4**.

31

## 32 **Discussion**

33 Activating mutations in the *KRAS* oncogene are known to trigger and sustain the  
34 development of tumors with different histology, being particularly frequent in lung, colon

1 and pancreatic cancer (1). The comparison between predicted and observed frequency of  
2 different mutant KRAS isoforms suggests the existence of tissue-specific mechanisms of  
3 biologic selection (14), however the connection between *KRAS*-mutant isoforms and  
4 therapeutic vulnerabilities has not been fully addressed yet. Very recently, the  
5 development of two specific inhibitors of the *KRAS-G12C* allele, sotorasib and adagrasib,  
6 which were granted accelerated FDA approval and breakthrough therapy designation  
7 (BTD), respectively, in patients with previously treated advanced NSCLC (10,38–41)  
8 highlighted the need to identify new isoform-specific therapeutic strategies. Here we  
9 demonstrate that KRAS isoforms are characterized by different oncogenic properties and  
10 therapeutic sensitivities in isogenic models, and that *KRAS*-mutant LUADs are highly  
11 heterogenous in terms of clinicopathologic, genomic, and transcriptomic features.

12 Studies have been inconsistent in reporting whether KRAS isoforms differ in terms  
13 of oncogenic properties, preferred signaling pathways, genomics and clinical outcomes in  
14 patients with LUAD (1). Previously, NSCLC patient samples and cell lines harboring KRAS  
15 G12A have been shown to primarily rely on phosphatidylinositol 3-kinase (PI3K) and  
16 mitogen-activated protein/extracellular signal-regulated kinase kinase (MEK) signaling,  
17 while those with mutant KRAS G12C or mutant KRAS G12V are more likely to activate  
18 Ral signaling and decrease growth factor-dependent Akt activation (19). Similarly, in  
19 KRAS overexpressing clones from human cell lines harboring the three most common  
20 *KRAS* mutations in NSCLC (G12C, G12V, and G12D), the expression of a specific KRAS  
21 isoforms induced a different sensitivity pattern to cytotoxic agents, such as cisplatin,  
22 pemetrexed and taxanes (42). Nonetheless, subsequent retrospective analysis yielded  
23 highly variable results in terms of clinical outcomes to either chemotherapy or PD-(L)1  
24 inhibition in patients with advanced NSCLC, as well as among those with resected tumors  
25 (19,42–43). However, data on KRAS co-mutation pattern have been underreported in  
26 these studies, therefore dissecting the biological differences among KRAS isoforms and  
27 their impact on clinical outcomes in patients with LUAD remains challenging.

28 To study the oncogenic properties of KRAS isoforms in a genetic-controlled system  
29 with the same genomic background, we generated isogenic models to dissect potential  
30 isoform-dependent therapeutic vulnerabilities. It has already been demonstrated that  
31 specific KRAS isoforms exhibit diverse biochemical and biological features *in vitro* and *in*  
32 *vivo* (19,21,25,44–45). However, the therapeutic impact of different KRAS alleles on  
33 treatment outcome has been only limitedly investigated so far (29,46,47). In our KRas<sup>lox</sup>  
34 KRAS<sup>MUT</sup> platform expressing the most frequent KRAS mutant alleles (G12C, G12D,

1 G12V, G12A, G13D and Q61H), we observed that G12D mutant cells show a significant  
2 growth advantage over the other mutants both *in vitro* and *in vivo* and are characterized,  
3 together with G12V, by the highest levels of GTP-bound fraction even in absence of  
4 upstream EGF stimulation.

5 To determine whether these differences translated in differential clinical outcomes  
6 we analyzed a large cohort of patients with LUAD. Although we did not identify significant  
7 differences in survival in patients with either early stage or advanced LUADs according to  
8 the different *KRAS* variants, we found that each *KRAS* isoform was associated with  
9 distinct clinicopathologic, genomic, and transcriptomic features, which may have impacted  
10 our ability to identify differences in outcomes. These data are in agreement with current  
11 knowledge confirming no significant differences in survival in LUAD patients according to  
12 specific *KRAS* mutant alleles. Still, *KRAS* isoform specificity could be used as a  
13 stratification criterion for better tailored therapies based on exclusive vulnerabilities. For  
14 instance, *KRAS* G12D mutated LUADs were more likely to develop in patients with no  
15 history of tobacco use. Consistently, tumors with *KRAS* G12D mutation had also the  
16 lowest TMB among *KRAS* isoforms, were less likely to harbor concurrent mutations in  
17 *STK11* or *KEAP1*, and had a marked downregulation of pathways involved in cellular  
18 senescence and chromosome maintenance. In contrast, the highest rate of concurrent  
19 mutations in *STK11* and *KEAP1* was identified among *KRAS* G13X mutated tumors.  
20 Concurrent mutations in *STK11* and *KEAP1* define unique subsets of *KRAS* mutant  
21 LUADs, with distinct immunophenotype and transcriptomic profile, and are generally  
22 characterized by significantly worse survival with both chemotherapy and PD-(L)1  
23 blockade (48,49). Consistently, we identified that G13X LUADs had a significant  
24 downregulation of pathways involved in innate and adaptative immunity, such as PD-1  
25 signaling, TCR signaling, INF $\gamma$  signaling, among others. It should be highlighted that gene  
26 expression profiling was performed NSCLC samples from the TCGA, rather than in our  
27 isogenic models. Therefore, these results should be interpreted in the context of the  
28 different genomic background which characterize each *KRAS* allele. Overall, our results  
29 support the hypothesis that in addition to the allele specific and intrinsic differences that  
30 we identified in our isogenic, each *KRAS* allele has a different spectrum of co-mutations  
31 and transcriptomic profiles in patient samples, which may not be dependent uniquely from  
32 *KRAS* alleles, but still have great biological relevance. Together, these results may have  
33 direct and immediate implications for the design and interpretation of pan*KRAS* or

1 multi/KRAS targeted therapies, as each individual allele has unique oncogenic properties  
2 and features.

3 To further dissect this question, we also investigated differential response to  
4 targeted therapy in KRAs<sup>lox</sup> KRAS<sup>MUT</sup> cells. As a proof of concept we chose MEK inhibitors,  
5 which showed promising results in Phase II clinical trial but failed to provide any significant  
6 benefit in addition to chemotherapy in KRAS-mutant LUAD in Phase III trial (4).  
7 Interestingly, retrospective analysis of Phase II trial showed a trend for KRAS G12C and  
8 G12V patients towards greater improvement in overall survival, progression free survival  
9 and overall response rate compared with other KRAS mutations (20). Indeed, we  
10 observed a consistently increased sensitivity of G12C and Q61H mutants with a panel of  
11 MEK inhibitors *in vitro*, which was maintained in a pre-clinical setting *in vivo* where G12C  
12 and Q61H implants showed greater tumor volume reduction compared to other mutants.  
13 This effect may partly rely on increased affinity to CRAF for some isoforms (29,50). Also,  
14 we validated the convenience of our pre-clinical tool in surveying combination therapies  
15 which can be easily tested in high-throughput screening, such as G12C/MEK inhibitors or  
16 SHP2/MEK inhibitors (30,31).

17 Our study has limitations inherent to the artificial generation of our isogenic model, and the  
18 retrospective assessment of clinical outcomes. However, we believe that our model does  
19 isolate the biological contribution of each isoform and allow the exploration of drug  
20 sensitivity in absence of potential confounding factors. This is of particular importance,  
21 given that our results indicate that each KRAS isoform is associated with unique co-  
22 mutation patterns and transcriptomic profiles which may affect drug sensitivity  
23 independently from KRAS intrinsic activity. Our results suggest that the use of specific  
24 genetically defined *in vitro* tools together with the development of large patient-derived  
25 datasets will facilitate the identification of potentially actionable vulnerabilities for KRAS  
26 mutant NSCLC.

27 In summary, this study indicates that KRAS isoforms are biologically and clinically  
28 heterogenous, and that such heterogeneity should be considered to optimize drug  
29 development strategies for KRAS mutant NSCLC.

30

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5

## 6 **Author contributions**

7 B.R., M.M.A., P.A.J. and C.A. designed the research strategy. B.R., J.S., J.J.O., X.W.,  
8 R.P., Y.E., M.L., H.H., J.L., Y.X., J.V.A., C.C., A.M., E.P., K.D., Y.C., E.R., M-J.N. and  
9 C.A. performed experiments and analyses. B.R., J.S., M.M.A., P.A.J. and C.A. wrote the  
10 manuscript. H.W., A.J.R., J.K., D.S. and P.C.G. helped to interpret results. All authors  
11 reviewed and approved the final manuscript.

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13

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## 15 **References**

- 16 1. Salgia R, Pharaon R, Mambetsariev I, Nam A, Sattler M. The improbable targeted  
17 therapy: KRAS as an emerging target in non-small cell lung cancer (NSCLC). *Cell*  
18 *Reports Med.* 2021.
- 19 2. Judd J, Abdel Karim N, Khan H, Naqash AR, Baca Y, Xiu J, et al. Characterization  
20 of KRAS Mutation Subtypes in Non-small Cell Lung Cancer. *Mol Cancer Ther.*  
21 2021;
- 22 3. Köhler J, Catalano M, Ambrogio C. Back to the bench? MEK and ERK inhibitors for  
23 the treatment of KRAS mutant lung adenocarcinoma. *Curr Med Chem.* 2017;24:1–  
24 17.
- 25 4. Jänne P, van den Heuvel M, Barlesi F, Cobo M, Mazieres J, Crinò L, et al.  
26 Selumetinib Plus Docetaxel Compared With Docetaxel Alone and Progression-Free  
27 Survival in Patients With KRAS-Mutant Advanced Non-Small Cell Lung Cancer: The  
28 SELECT-1 Randomized Clinical Trial. *JAMA.* 2017;317:1844–53.
- 29 5. Planchard D, Popat S, Kerr K, Novello S, Smit EF, Faivre-Finn C, et al. Metastatic  
30 non-small cell lung cancer: ESMO Clinical Practice Guidelines for diagnosis,  
31 treatment and follow-up. *Ann Oncol.* 2018;
- 32 6. Ambrogio C, López-Gómez G, Falcone M, Vidal A, Nadal E, Crosetto N, et al.  
33 Combined inhibition of DDR1 and Notch signaling is a therapeutic strategy for  
34 KRAS-driven lung adenocarcinoma. *Nat Med.* 2016;22:270–7.



- 1 7. Manchado E, Weissmueller S, Morris JP, Chen C-C, Wullenkord R, Lujambio A, et  
2 al. A combinatorial strategy for treating KRAS-mutant lung cancer. *Nature*.  
3 2016;534:647–51.
- 4 8. Awad MM, Liu S, Rybkin II, Arbour KC, Dilly J, Zhu VW, et al. Acquired Resistance  
5 to KRAS G12C Inhibition in Cancer . *N Engl J Med*. 2021;384.
- 6 9. Skoulidis F, Li BT, Dy GK, Price TJ, Falchook GS, Wolf J, et al. Sotorasib for Lung  
7 Cancers with KRAS p.G12C Mutation . *N Engl J Med*. 2021;384.
- 8 10. Riely GJ, Ou S-HI, Rybkin I, Spira A, Papadopoulos K, Sabari JK, et al. 99O\_PR  
9 KRYSTAL-1: Activity and preliminary pharmacodynamic (PD) analysis of adagrasib  
10 (MRTX849) in patients (Pts) with advanced non–small cell lung cancer (NSCLC)  
11 harboring KRASG12C mutation. *J Thorac Oncol*. 2021;16.
- 12 11. Santos E, Martin-Zanca D, Reddy EP, Pierotti M a, Della Porta G, Barbacid M.  
13 Malignant activation of a K-ras oncogene in lung carcinoma but not in normal tissue  
14 of the same patient. *Science*. 1984;223:661–4.
- 15 12. Ambrogio C, Köhler J, Zhou Z, Wang H, Paranal R, Li J, et al. KRAS Dimerization  
16 Impacts MEK Inhibitor Sensitivity and Oncogenic Activity of Mutant KRAS. *Cell*.  
17 2018;S0092-8674.
- 18 13. Zhou Y, Prakash P, Liang H, Cho KJ, Gorfe AA, Hancock JF. Lipid-Sorting  
19 Specificity Encoded in K-Ras Membrane Anchor Regulates Signal Output. *Cell*.  
20 2017;168:239-251.e16.
- 21 14. Cook J, Melloni G, Gulhan D, Park P, Haigis KM. The origins and genetic  
22 interactions of KRAS mutations are allele- and tissue-specific. *Nat Commun*. 2021;
- 23 15. Hood FE, Klinger B, Newlaczyl AU, Sieber A, Dorel M, Oliver SP, et al. Isoform-  
24 specific Ras signaling is growth factor dependent. *Mol Biol Cell*. 2019;
- 25 16. Tang R, Shuldiner EG, Kelly M, Murray CW, Hebert JD, Andrejka L, et al.  
26 Multiplexed identification of RAS paralog imbalance as a driver of lung cancer  
27 growth. *bioRxiv*. 2021;
- 28 17. Tejpar S, Celik I, Schlichting M, Sartorius U, Bokemeyer C, Van Cutsem E.  
29 Association of KRAS G13D tumor mutations with outcome in patients with  
30 metastatic colorectal cancer treated with first-line chemotherapy with or without  
31 cetuximab. *J Clin Oncol*. 2012;
- 32 18. Burd CE, Liu W, Huynh M V., Waqas MA, Gillahan JE, Clark KS, et al. Mutation-  
33 specific RAS oncogenicity explains NRAS codon 61 selection in melanoma. *Cancer*  
34 *Discov*. 2014;

- 1 19. Ihle NT, Byers LA, Kim ES, Saintigny P, Lee JJ, Blumenschein GR, et al. Effect of  
2 KRAS oncogene substitutions on protein behavior: Implications for signaling and  
3 clinical outcome. *J Natl Cancer Inst.* 2012;104:228–39.
- 4 20. Jänne P a, Smith I, McWalter G, Mann H, Dougherty B, Walker J, et al. Impact of  
5 KRAS codon subtypes from a randomised phase II trial of selumetinib plus  
6 docetaxel in KRAS mutant advanced non-small-cell lung cancer. *Br J Cancer*  
7 [Internet]. 2015;113:199–203. Available from:  
8 [http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=4506393&tool=pmcentrez](http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=4506393&tool=pmcentrez&rendertype=abstract)  
9 &rendertype=abstract
- 10 21. Hammond DE, Mageean CJ, Rusilowicz E V., Wickenden JA, Clague MJ, Prior IA.  
11 Differential reprogramming of isogenic colorectal cancer cells by distinct activating  
12 KRAS mutations. *J Proteome Res.* 2015;
- 13 22. Stolze B, Reinhart S, Bullinger L, Fröhling S, Scholl C. Comparative analysis of  
14 KRAS codon 12, 13, 18, 61, and 117 mutations using human MCF10A isogenic cell  
15 lines. *Sci Rep* [Internet]. 2015;5:8535. Available from:  
16 <http://www.nature.com/srep/2015/150223/srep08535/full/srep08535.html>
- 17 23. Brubaker DK, Paulo JA, Sheth S, Poulin EJ, Popow O, Joughin BA, et al.  
18 Proteogenomic Network Analysis of Context-Specific KRAS Signaling in Mouse-to-  
19 Human Cross-Species Translation. *Cell Syst.* 2019;9.
- 20 24. Drosten M, Dhawahir A, Sum EYMM, Urosevic J, Lechuga CG, Esteban LM, et al.  
21 Genetic analysis of Ras signalling pathways in cell proliferation, migration and  
22 survival. *EMBO J* [Internet]. 2010;29:1091–104. Available from:  
23 <http://dx.doi.org/10.1038/emboj.2010.7>
- 24 25. Ricciuti B, Jones G, Severgnini M, Alessi JV, Recondo G, Lawrence M, Forshew T,  
25 Lydon C, Nishino M, Cheng M, Awad M. Early plasma circulating tumor DNA  
26 (ctDNA) changes predict response to first-line pembrolizumab-based therapy in non-  
27 small cell lung cancer (NSCLC). *J Immunother Cancer.* 2021;9(3):e001504
- 28 26. Winters I, Chiou S, Paulk N, McFarland C, Lalgudi P, Ma R, et al. Multiplexed in vivo  
29 homology-directed repair and tumor barcoding enables parallel quantification of  
30 Kras variant oncogenicity. *Nat Commun.* 2017;1–16.
- 31 27. Garnett MJ, Edelman EJ, Heidorn SJ, Greenman CD, Dastur A, Lau KW, et al.  
32 Systematic identification of genomic markers of drug sensitivity in cancer cells.  
33 *Nature* [Internet]. 2012;483:570–5. Available from:  
34 <http://europepmc.org/articles/PMC3349233>

- 1 28. Li S, Liu S, Deng J, Akbay E, Hai J, Ambrogio C, et al. Assessing Therapeutic  
2 Efficacy of MEK Inhibition in a KRAS G12C-Driven Mouse Model of Lung Cancer.  
3 Clin Cancer Res. 2018;
- 4 29. Zhou Z-W, Ambrogio C, Bera AK, Li Q, Li X-X, Li L, et al. KRAS<sup>Q61H</sup> preferentially  
5 signals through MAPK in a RAF dimer-dependent manner in non-small cell lung  
6 cancer. Cancer Res. 2020;80.
- 7 30. Mainardi S, Mulero-Sánchez A, Prahallad A, Germano G, Bosma A, Krimpenfort P,  
8 et al. SHP2 is required for growth of KRAS-mutant non-small-cell lung cancer in vivo  
9 letter. Nat Med. 2018;
- 10 31. Ruess DA, Heynen GJ, Ciecieski KJ, Ai J, Berninger A, Kabacaoglu D, et al. Mutant  
11 KRAS-driven cancers depend on PTPN11/SHP2 phosphatase. Nat Med [Internet].  
12 Springer US; 2018;24:954–60. Available from: [http://dx.doi.org/10.1038/s41591-](http://dx.doi.org/10.1038/s41591-018-0024-8)  
13 [018-0024-8](http://dx.doi.org/10.1038/s41591-018-0024-8)
- 14 32. Gebregiworgis T, Kano Y, St-Germain J, Radulovich N, Udaskin M, Mentes A, et al.  
15 The Q61H mutation decouples KRAS from upstream regulation and renders cancer  
16 cells resistant to SHP2 inhibitors. Nat Commun. 2021;
- 17 33. El Osta B, Behera M, Kim S, Berry LD, Sica G, Pillai RN, et al. Characteristics and  
18 Outcomes of Patients With Metastatic KRAS-Mutant Lung Adenocarcinomas: The  
19 Lung Cancer Mutation Consortium Experience. J Thorac Oncol. 2019;14.
- 20 34. Dogan S, Shen R, Ang DC, Johnson ML, D'Angelo SP, Paik PK, et al. Molecular  
21 epidemiology of EGFR and KRAS mutations in 3,026 lung adenocarcinomas: Higher  
22 susceptibility of women to smoking-related KRAS-mutant cancers. Clin Cancer Res.  
23 2012;18.
- 24 35. Riely GJ, Kris MG, Rosenbaum D, Marks J, Li A, Chitale DA, et al. Frequency and  
25 distinctive spectrum of KRAS mutations in never smokers with lung  
26 adenocarcinoma. Clin Cancer Res. 2008;14.
- 27 36. Skoulidis F, Goldberg ME, Greenawalt DM, Hellmann MD, Awad MM, Gainor JF, et  
28 al. STK11/LKB1 mutations and PD-1 inhibitor resistance in KRAS-mutant lung  
29 adenocarcinoma. Cancer Discov. 2018;8.
- 30 37. Chen X, Su C, Ren S, Zhou C, Jiang T. Pan-cancer analysis of KEAP1 mutations as  
31 biomarkers for immunotherapy outcomes. Ann Transl Med. 2020;8.
- 32 38. Li S, Balmain A, Counter CM. A model for RAS mutation patterns in cancers: finding  
33 the sweet spot. Nat. Rev. Cancer. 2018.
- 34 39. Canon J, Rex K, Saiki A, Mohr C, Cooke K, Bagal D, et al. The clinical KRAS(G12C)

- 1 inhibitor AMG 510 drives anti-tumour immunity. *Nature*. 2019;
- 2 40. Hallin J, Engstrom L, Hargis L, Calinisan A, Aranda R, Briere D, et al. The KRAS  
3 G12C Inhibitor, MRTX849, Provides Insight Toward Therapeutic Susceptibility of  
4 KRAS Mutant Cancers in Mouse Models and Patients. *Cancer Discov*. 2019;
- 5 41. Hong DS, Fakih MG, Strickler JH, Desai J, Durm GA, Shapiro GI, et al. KRAS-G12C  
6 inhibition with sotorasib in advanced solid tumors. *N Engl J Med*. 2020;
- 7 42. Garassino M, Marabese M, Rusconi P, Rulli E, Martelli O, Farina G, et al. Different  
8 types of K-Ras mutations could affect drug sensitivity and tumour behaviour in non-  
9 small-cell lung cancer. *Ann Oncol*. 2011;
- 10 43. Shepherd FA, Domerg C, Hainaut P, Jänne P, Pignon J, Graziano S, et al. Pooled  
11 analysis of the prognostic and predictive effects of KRAS mutation status and KRAS  
12 mutation subtype in early-stage resected non-small-cell lung cancer in four trials of  
13 adjuvant chemotherapy. *J Clin Oncol*. 2013;31:2173–81.
- 14 44. Hunter JC, Manandhar A, Carrasco MA, Gurbani D, Gondi S, Westover KD.  
15 Biochemical and Structural Analysis of Common Cancer-Associated KRAS  
16 Mutations. *Mol Cancer Res [Internet]*. 2015;13:1325–35. Available from:  
17 <http://mcr.aacrjournals.org/cgi/doi/10.1158/1541-7786.MCR-15-0203>
- 18 45. Zafra MP, Parsons MJ, Kim J, Alonso-Curbelo D, Goswami S, Schatoff EM, et al.  
19 An in vivo kras allelic series reveals distinct phenotypes of common oncogenic  
20 variants. *Cancer Discov*. 2020;10.
- 21 46. De Roock W, Jonker DJ, Di Nicolantonio F, Sartore-Bianchi A, Tu D, Siena S, et al.  
22 Association of KRAS p.G13D mutation with outcome in patients with chemotherapy-  
23 refractory metastatic colorectal cancer treated with cetuximab. *JAMA - J Am Med*  
24 *Assoc*. 2010;304.
- 25 47. Bournet B, Muscari F, Buscail C, Assenat E, Barthelet M, Hammel P, et al. KRAS  
26 G12D Mutation Subtype Is A Prognostic Factor for Advanced Pancreatic  
27 Adenocarcinoma. *Clin Transl Gastroenterol*. 2016;7.
- 28 48. Skoulidis F, Byers LA, Diao L, Papadimitrakopoulou VA, Tong P, Izzo J, et al. Co-  
29 occurring genomic alterations define major subsets of KRAS-mutant lung  
30 adenocarcinoma with distinct biology, immune profiles, and therapeutic  
31 vulnerabilities. *Cancer Discov*. 2015;5:861–78.
- 32 49. Ricciuti B, Arbour K, Lin J, Vajdi A, Vokes N, Hong L, et al. Diminished Efficacy of  
33 Programmed Death-(Ligand)1 Inhibition in STK11- and KEAP1-Mutant Lung  
34 Adenocarcinoma Is Affected by KRAS Mutation Status. *J Thorac Oncol*. 2021;

1 50. Mysore VP, Zhou ZW, Ambrogio C, Li L, Kapp JN, Lu C, et al. A structural model of  
2 a Ras–Raf signalosome. Nat Struct Mol Biol. 2021;28.

3

#### 4 **Figure Legends**

5 **Figure 1:** (A) schematic representation of the KRas<sup>lox</sup> KRAS<sup>MUT</sup> system (top panel). *HRas*<sup>-</sup>  
6 <sup>-/-</sup>; *NRas*<sup>-/-</sup>; *KRAS*<sup>lox/lox</sup> mouse embryonic fibroblasts were infected with 5-MOI of  
7 retroviruses encoding for different human HA-tagged KRAS mutants, selected with  
8 puromycin (1μg/ml), treated with 4OHT (600nM) for at least 10 days and probed by  
9 Western Blot with the indicated antibodies (bottom panel). (B) KRas<sup>lox</sup> KRAS<sup>MUT</sup> cells were  
10 treated with 4OHT for 10 days and probed by Western Blot with the indicated antibodies.  
11 Results are representative of one of three similar experiments. (C) KRas-GTP levels and  
12 activation of downstream pMEK signaling in KRas<sup>lox</sup> KRAS<sup>MUT</sup> cells in 0.1% FBS medium  
13 upon stimulation with EGF (50 ng/mL). Results are representative of one of three similar  
14 experiments. (D) Growth rates of KRas<sup>lox</sup> KRAS<sup>MUT</sup> cells in 10% or 0.5% fetal bovine  
15 serum (FBS) medium in 2D conditions as assessed by IncuCyte measurements (left  
16 panels,  $p < 0.0001$  by unpaired Student's t test). Results are representative of one of three  
17 similar experiments. Growth of KRas<sup>lox</sup> KRAS<sup>MUT</sup> organoids was monitored by CTG assay.  
18 ANOVA analysis followed by Tukey's multiple comparisons post-test was used for  
19 statistical analysis ( $p < 0.0001$ ). (E) KRas<sup>lox</sup> KRAS<sup>MUT</sup> cells ( $1 \times 10^6$ ) were injected into the  
20 tail vein of nude mice ( $n=3$  per isoform). Animals were sacrificed after one month and lung  
21 colonization checked by serial sections stained with hematoxylin and eosin. The number  
22 of animals with evidence of lung colonization is indicated in the table. Scale bar: 100μM.

23

24 **Figure 2:** (A) Comparison of IC50 values to MEK inhibitor selumetinib between KRas<sup>lox</sup>  
25 KRAS<sup>MUT</sup> cells expressing different KRAS isoforms. Error bars represent mean  $\pm$  SD.  
26 Results are representative of one of three similar experiments. The table indicates cell  
27 lines ranked by IC50 values (lowest to highest). (B) Log GR50 values for selumetinib and  
28 trametinib are shown in the box plot. The numbers and vertical lines go through each box  
29 indicate the median. (C) KRas<sup>lox</sup> KRAS<sup>MUT</sup> cells were seeded and treated with the  
30 indicated concentrations of selumetinib for 12 hours and then probed by Western Blot with  
31 the indicated antibodies. Results are representative of one of three similar experiments.  
32 (D) KRas<sup>lox</sup> KRAS<sup>MUT</sup> cells ( $2 \times 10^6$ ) were injected subcutaneously with Matrigel (1:1) into  
33 nude mice. Mice with palpable tumors were randomized into two cohorts ( $n=6$ ) and treated  
34 with vehicle or selumetinib (50mg/kg) daily by oral gavage for one week. Tumor size was

1 measured every day with a caliper. Mean fold change in tumor volume relative to initial  
2 tumor volume is shown. Error bars represent mean  $\pm$  SD.

3  
4 **Figure 3:** (A) Comparison of IC50 values to the selective G12C inhibitor sotorasib  
5 between KRas<sup>lox</sup> KRAS<sup>MUT</sup> cells expressing different KRAS isoforms. Error bars represent  
6 mean  $\pm$  SD. Results are representative of one of three similar experiments. (B) KRas<sup>lox</sup>  
7 KRAS<sup>G12C</sup> cells ( $2 \times 10^6$ ) were injected subcutaneously with Matrigel (1:1) into nude mice.  
8 Mice with palpable tumors were randomized into four cohorts and treated with vehicle,  
9 selumetinib (50mg/kg), sotorasib (50mg/kg) or combo treatment daily by oral gavage.  
10 Tumor size was measured every day with a caliper. Tumor volume change relative to  
11 initial tumor volume is shown for individual tumors. (C) Bar graph showing average tumor  
12 volume fold change to baseline at end point. Error bars represent mean  $\pm$  SD. (D)  
13 Waterfall plot showing individual tumor volume change from baseline (%) at end point. (E)  
14 Macroscopic photographs after 10 days of treatment and tumor volume tracking of KRas<sup>lox</sup>  
15 KRAS<sup>G12C</sup> cells.

16  
17 **Figure 4:** Overall survival in (A) all comers with non-squamous NSCLC, and (B) stage I-  
18 III, and (C) stage IV disease according to KRAS mutation status. Overall survival in (D) all  
19 comers with non-squamous NSCLC, and (E) stage I-III, and (F) stage IV disease  
20 according to KRAS alleles.

21  
22 **Figure 5:** (A) OncoPrint plot of KRAS mutant LUADs in the GENIE v9.0 dataset. (B)  
23 Volcano plots showing co-mutation patterns in KRAS G12C, G12D, G12V, G12A, G13X,  
24 and G61X compared to KRAS wild type LUADs. Gene ontology analysis showing (C)  
25 down- and (D) up-regulated pathways in KRAS G12C, G12D, G12V, G12A, G13X, and  
26 G61X compared to KRAS wild type LUADs.

27

Figure 1

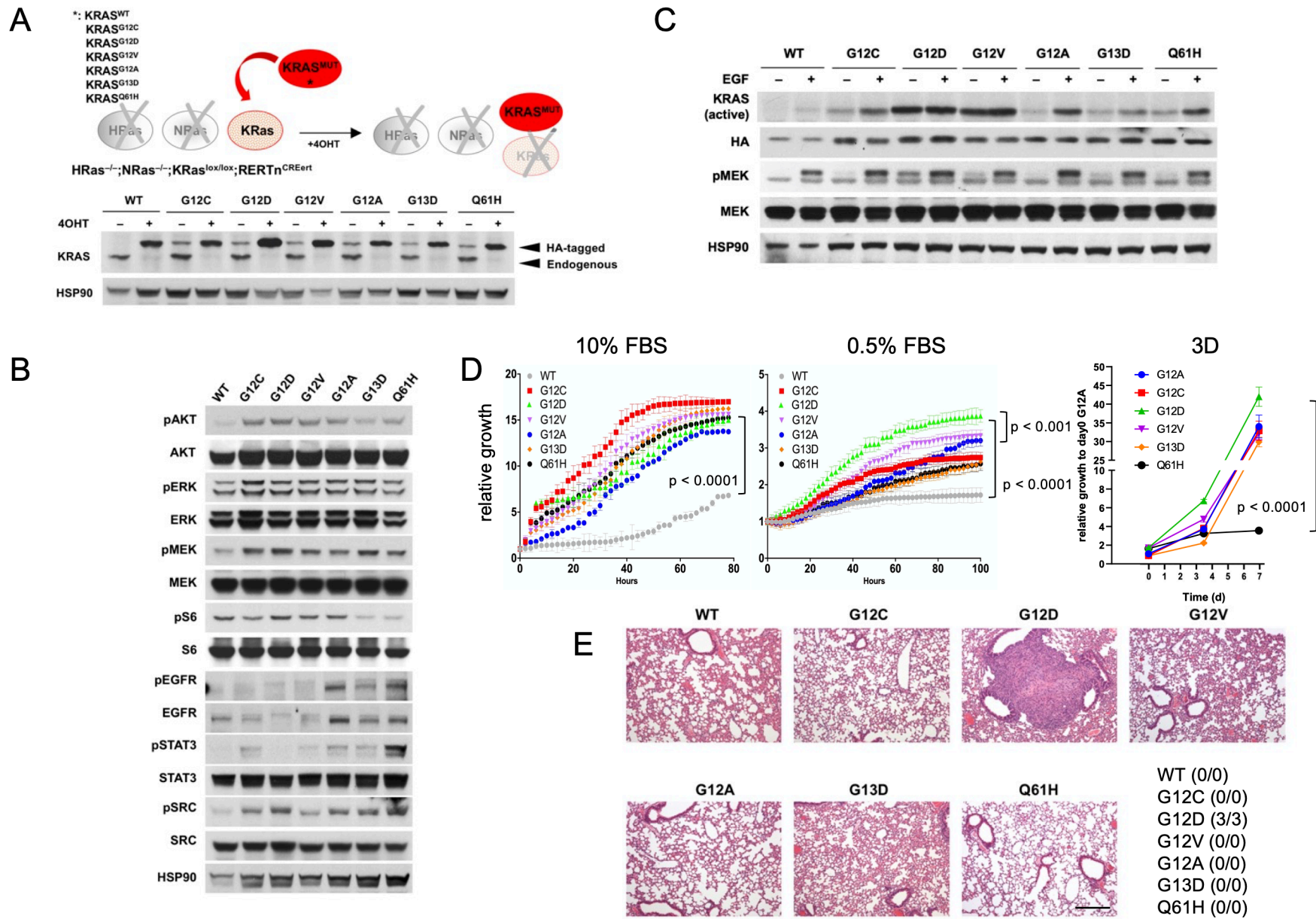


Figure 2

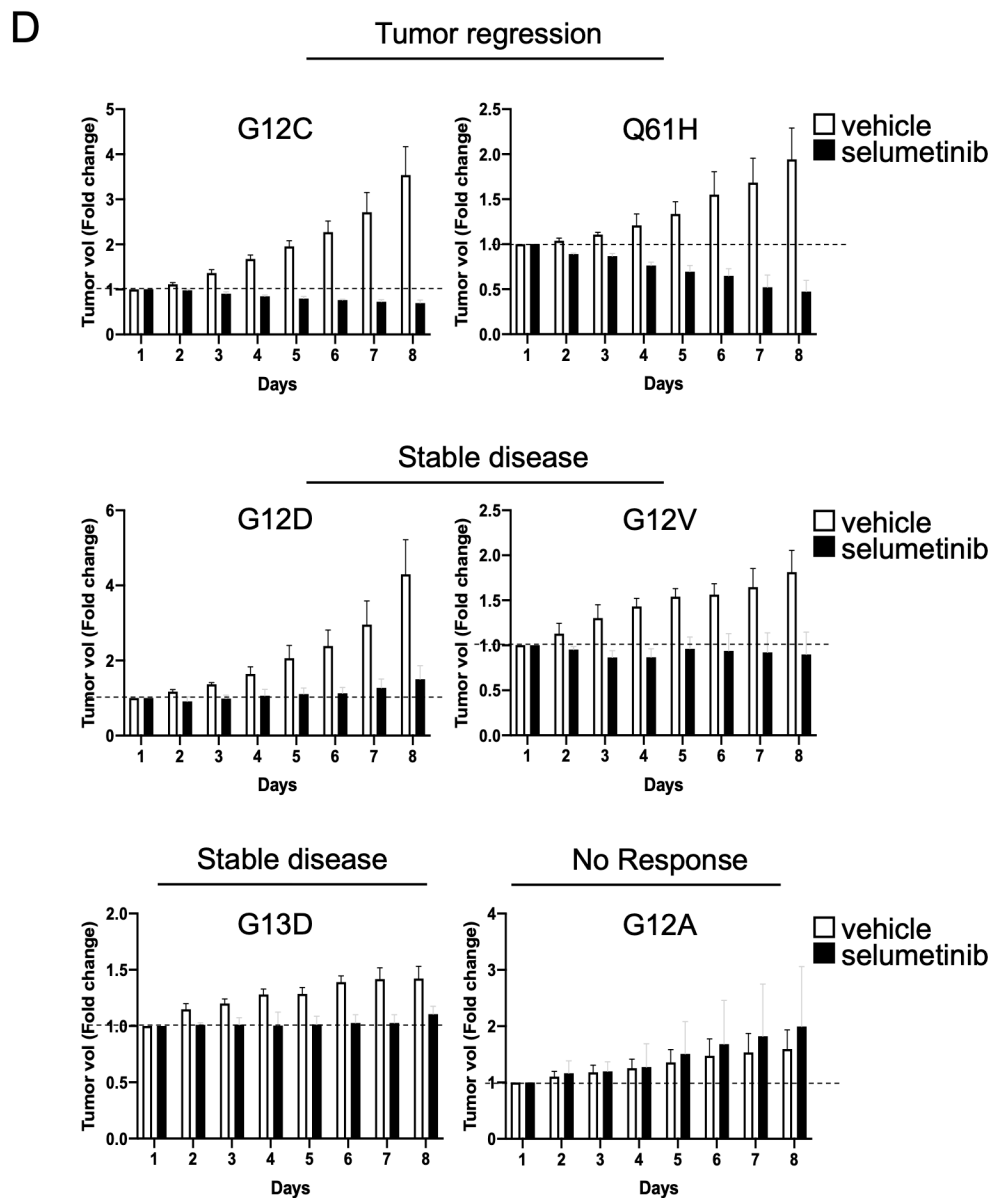
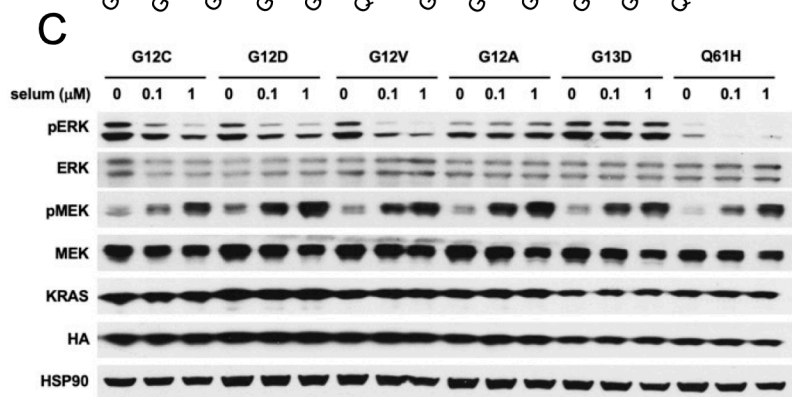
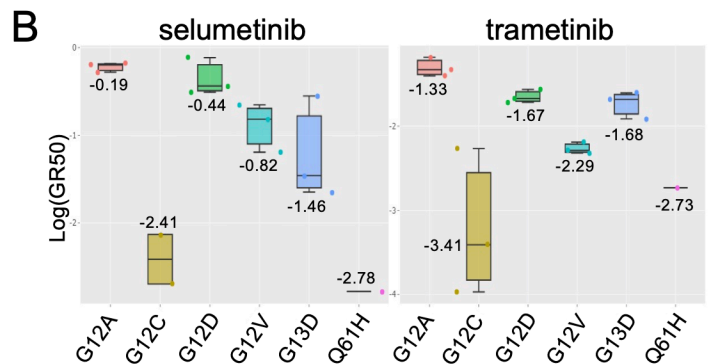
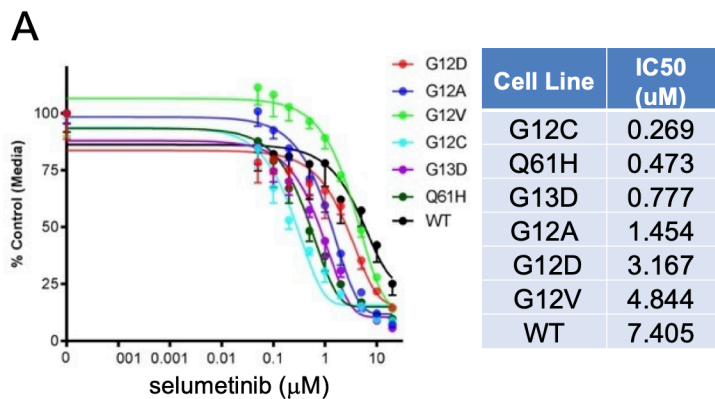




Figure 3

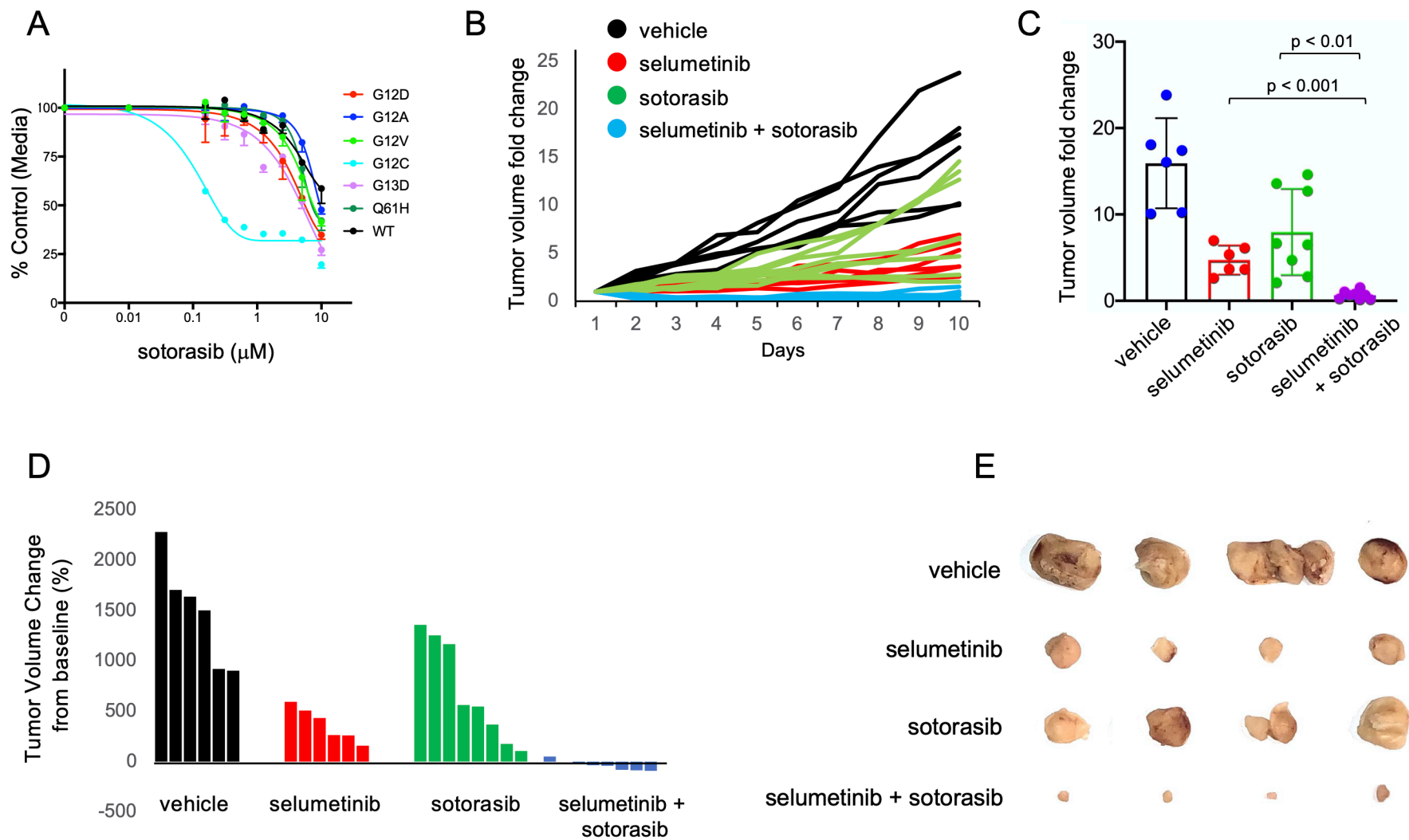


Figure 4

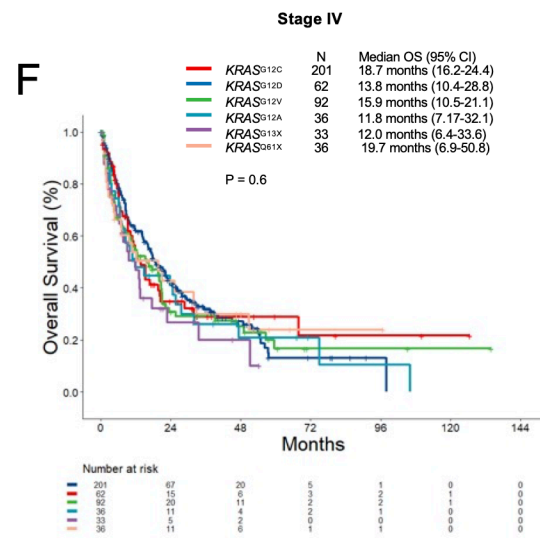
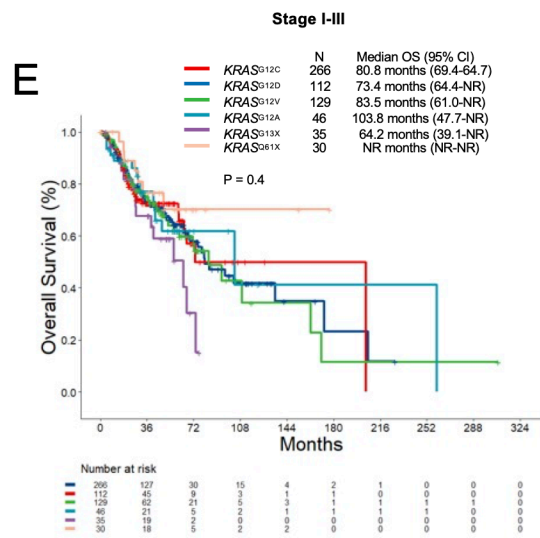
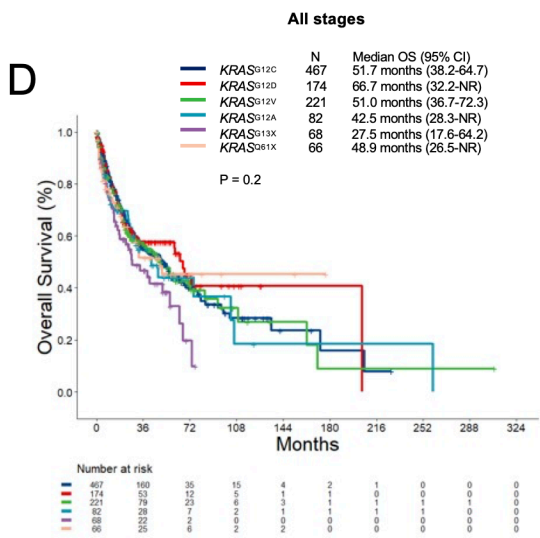
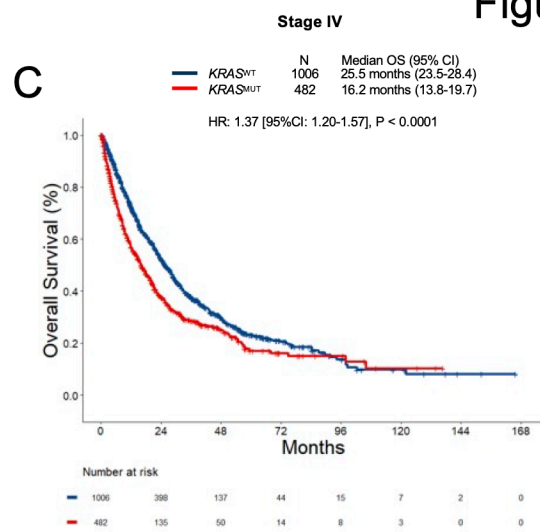
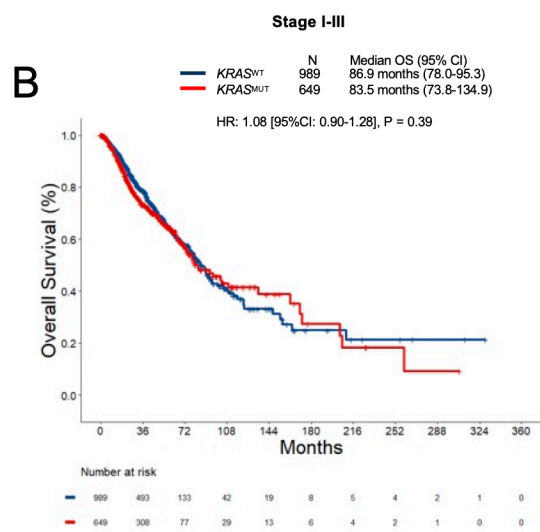
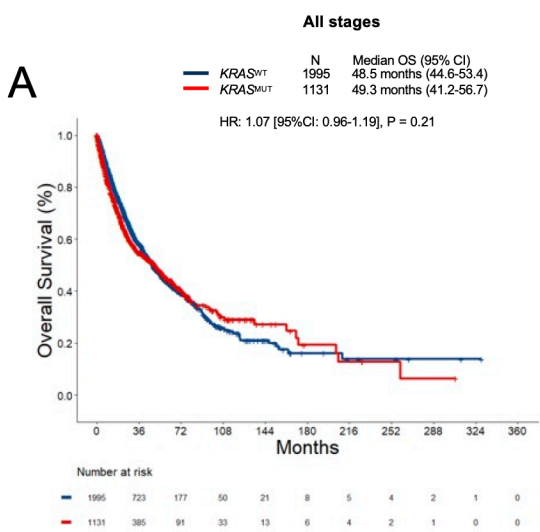


Figure 5

