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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**

3 *Biagio Ricciuti^{1,*}, Jieun Son^{2,*}, Jeffrey J. Okoro², Alessia Mira³, Enrico Patrucco³, Yoonji*
4 *Eum², Xinan Wang^{4,5}, Raymond Parana², Haiyun Wang⁶, Mika Lin², Heidi M. Haikala²,*
5 *Jiaqi Li², Yue Xu⁶, Joao Victor Alessi¹, Chhayheng Chhoeu⁷, Amanda J. Redig¹, Jens*
6 *Köhler², Kshiti H. Dholakia^{1,2}, Yunhan Chen², Elodie Richard⁸, Marie-Julie Nokin⁹, David*
7 *Santamaria⁹, Prafulla C. Gokhale⁷, Mark M. Awad^{1,§}, Pasi A. Jänne^{1,2,§}, Chiara*
8 *Ambrogio^{2,3,§}*

9
10 ¹*Lowe Center for Thoracic Oncology, Dana-Farber Cancer Institute, Harvard Medical School, Boston, USA*

11 ²*Department of Medical Oncology, Dana-Farber Cancer Institute and Harvard Medical School, Boston, USA*

12 ³*Department of Molecular Biotechnology and Health Sciences, Molecular Biotechnology Center, University*
13 *of Torino, Torino, Italy*

14 ⁴*Harvard Graduate School of Arts and Sciences, Harvard University, Cambridge, USA*

15 ⁵*Department of Environmental Health, Harvard T.H. Chan School of Public Health, Harvard University,*
16 *Boston, USA*

17 ⁶*School of Life Sciences and Technology, Tongji University, Shanghai 200092, China*

18 ⁷*Experimental Therapeutics Core and Belfer Center for Applied Cancer Science, Dana-Farber Cancer*
19 *Institute, Boston, USA*

20 ⁸*Institut Bergonié, INSERM U1218, ACTION Laboratory, Bordeaux, France*

21 ⁹*University of Bordeaux, INSERM U1218, ACTION Laboratory, IECB, Pessac, France*

22
23 **These authors contributed equally*

24 *§Co-senior authors*

25
26 *Address correspondence to:*

27 *-Chiara Ambrogio, Department of Molecular Biotechnology and Health Sciences, Molecular Biotechnology*
28 *Center, Via Nizza 10126 Torino, Italy. Phone: 011-6703222. Email: chiara.ambrogio@unito.it*

29 *-Pasi A. Jänne, Lowe Center for Thoracic Oncology Dana Farber Cancer Institute, 450 Brookline Avenue,*
30 *LC4114 Boston, MA 02215. Phone: 617-632-6036. Email: pasi_janne@dfci.harvard.edu*

31 *-Mark M.Awad, Dana-Farber Cancer Institute and Harvard Medical Center, 450 Brookline Avenue, Dana*
32 *1240F, Boston, MA 02215. Phone: 617-632-3468. Email: mark_awad@dfci.harvard.edu*

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^{lox} KRAS^{MUT} cells*

5 KRas^{lox} KRAS^{MUT} cells were generated as previously described (12). Briefly, KRAS^{MUT}
6 retroviral constructs were created by point mutagenesis from pBABE HA-tagged KRAS^{WT}
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*^{-/-}; *NRas*^{-/-}; *KRas*^{lox/lox} 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 (1x10³) 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 (1x10³) 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% O₂ 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 IC₅₀ 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 (1×10^3) 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 μ M to 15 μ M and from 0.5 μ M
18 to 15 μ 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 μ g) were incubated with 30 μ 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 μ 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*^{nu} mice (females, 8-week-old) were purchased from Charles River. For lung
15 colonization assays, KRas^{lox} KRAS^{MUT} cells (1×10^6) were injected tail vein as single-cell
16 suspension in 200 μ 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^{lox}
18 KRAS^{MUT} cells (2×10^6) 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^{lox} KRAS^{MUT} cells were transduced with pFUGW-Pol2-ffLuc2-eGFP (Addgene
25 plasmid #71394) and FACS-sorted for GFP expression. KRas^{lox} KRAS^{MUT}-luciferase cells
26 (2×10^6) were injected in the tail vein of 8-weeks old NCr nude mice (Taconic Biosciences,
27 NY) as single-cell suspension in 200 μ 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^{lox}KRAS^{MUT} 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*^{-/-}; *NRas*^{-/-}; *KRAS*^{lox/lox} mouse embryonic fibroblasts (MEFs) (24). In
5 these cells, *KRAS*^{lox/lox} alleles excision is controlled by a resident 4-hydroxytamoxifen
6 (4OHT)-dependent CRE^{ERT2} recombinase. We transduced *HRas*^{-/-}; *NRas*^{-/-}; *KRAS*^{lox/lox}
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^{lox} KRAS^{MUT} 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^{GTP} 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^{lox} KRAS^{MUT} 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^{lox} KRAS^{MUT} 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^{lox} KRAS^{MUT} 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^{lox} KRAS^{MUT} 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^{G12C} 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^{lox}
21 KRAS^{G12C} 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*^{MUT})
8 occurred in 1131 cases (36.2%), while the remaining 1995 cases (63.8%) had a *KRAS*
9 wild type (*KRAS*^{WT}) 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*^{WT} cases, patients with *KRAS*^{MUT} 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*^{WT} versus
17 *KRAS*^{MUT} 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*^{WT} 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*^{MUT} and *KRAS*^{WT} 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*^{MUT} versus *KRAS*^{WT} 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 γ , 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^{lox}
34 KRAS^{MUT} 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 γ 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^{lox} KRAS^{MUT} 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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3

4 **Figure Legends**

5 **Figure 1:** (A) schematic representation of the KRas^{lox} KRAS^{MUT} system (top panel). *HRas*⁻
6 ^{-/-}; *NRas*^{-/-}; *KRAS*^{lox/lox} 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^{lox} KRAS^{MUT} 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^{lox} KRAS^{MUT} 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^{lox} KRAS^{MUT} 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^{lox} KRAS^{MUT} 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^{lox} KRAS^{MUT} cells (1×10^6) 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^{lox}
25 KRAS^{MUT} 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^{lox} KRAS^{MUT} 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^{lox} KRAS^{MUT} cells (2×10^6) 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^{lox} KRAS^{MUT} cells expressing different KRAS isoforms. Error bars represent
6 mean \pm SD. Results are representative of one of three similar experiments. (B) KRas^{lox}
7 KRAS^{G12C} cells (2×10^6) 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^{lox}
15 KRAS^{G12C} 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

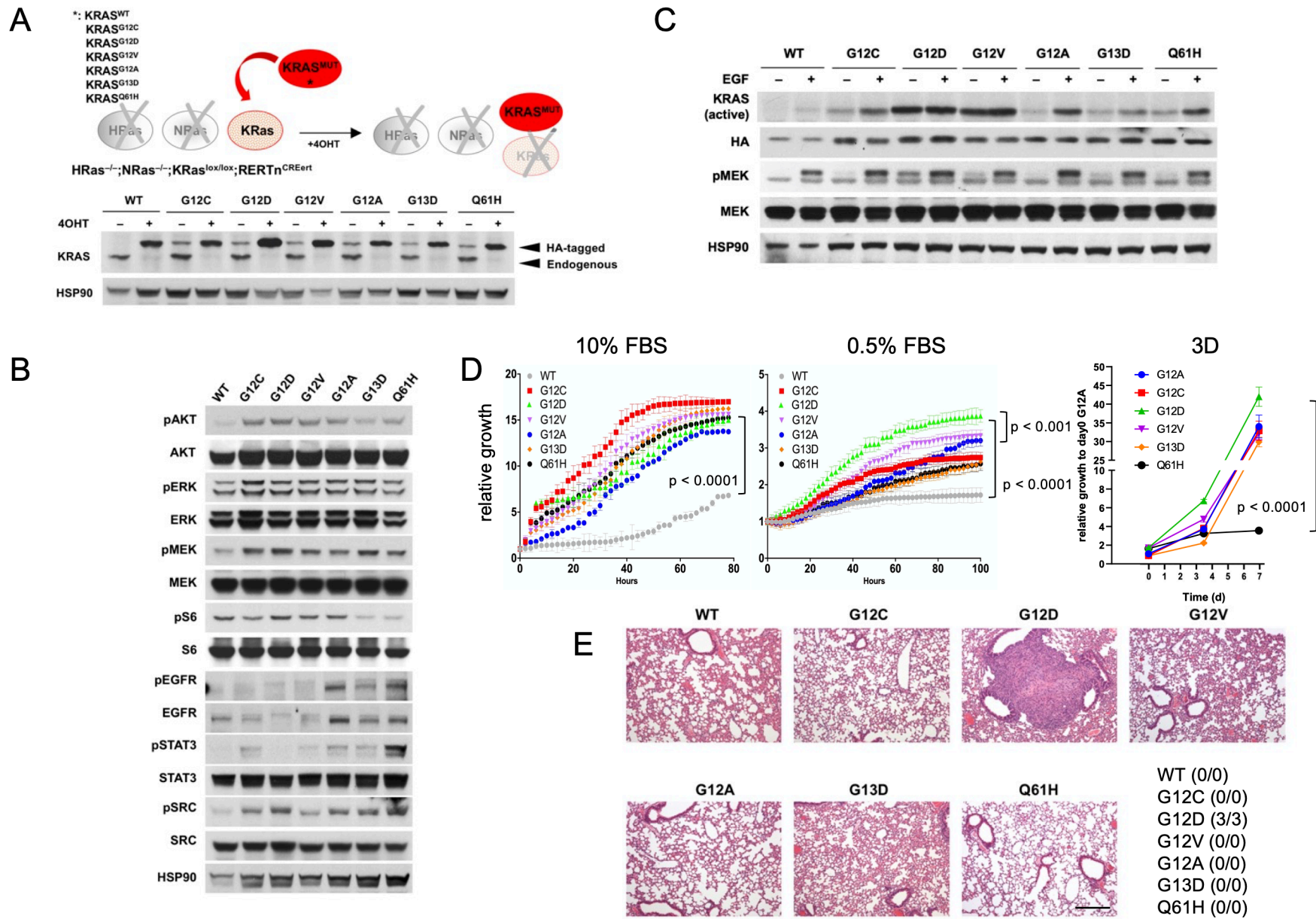


Figure 2

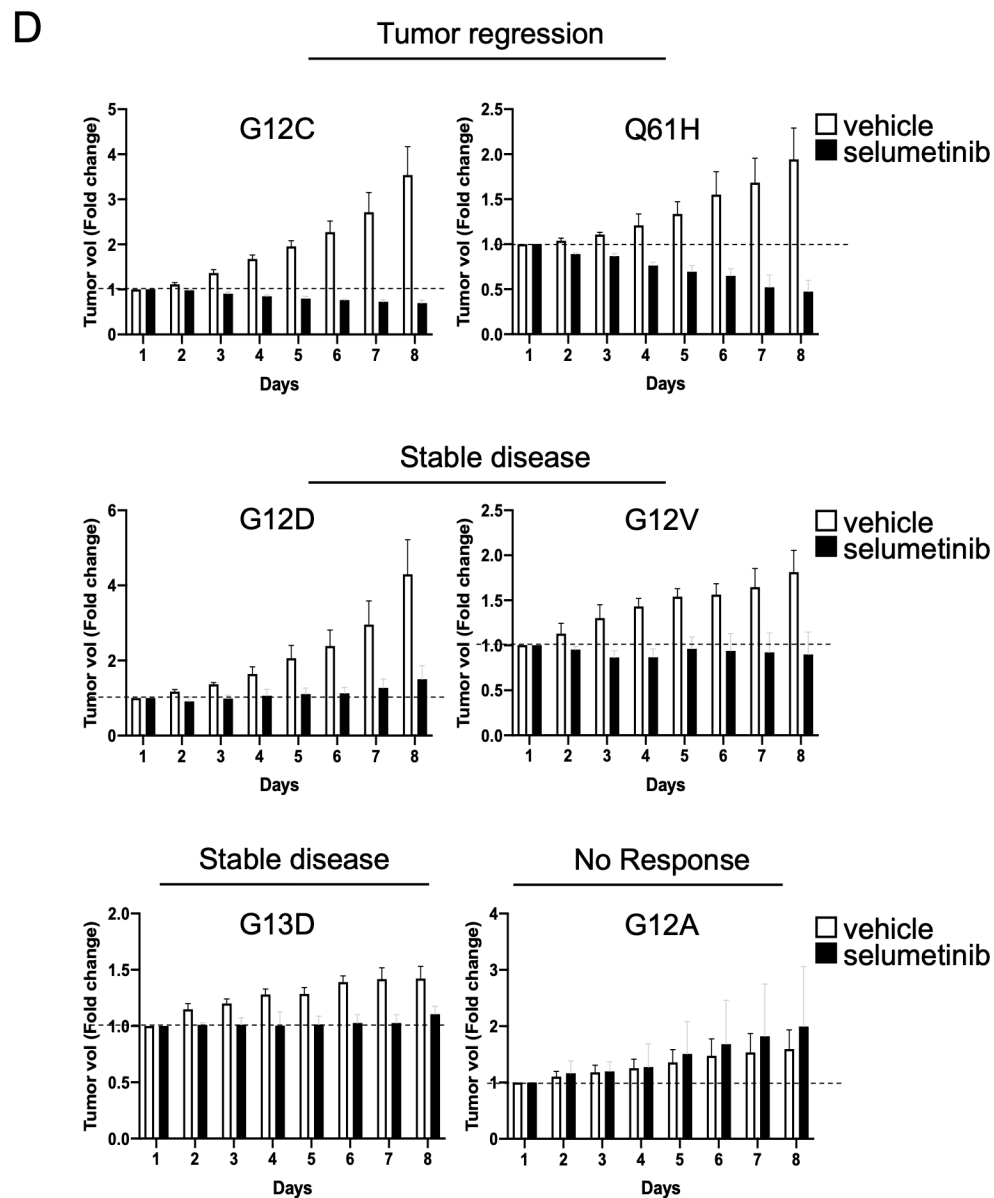
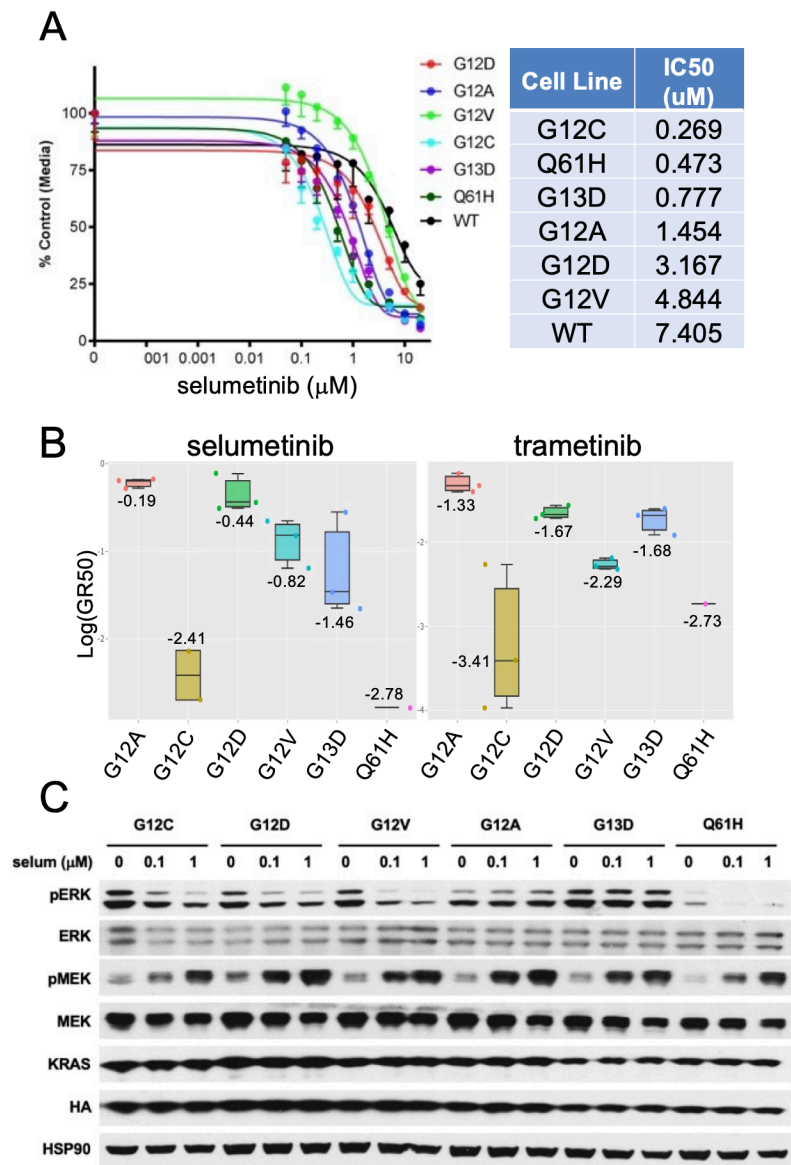


Figure 3

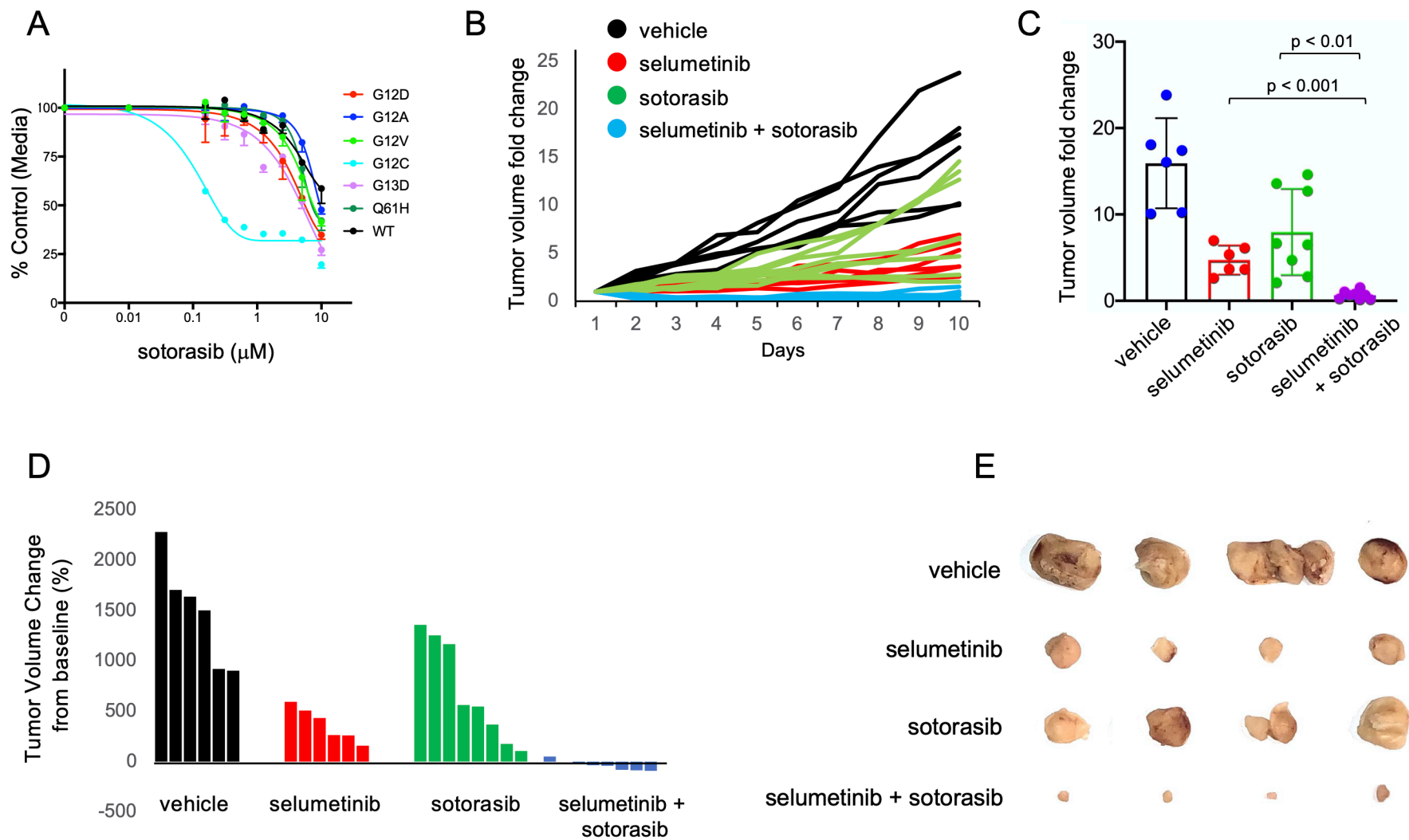


Figure 4

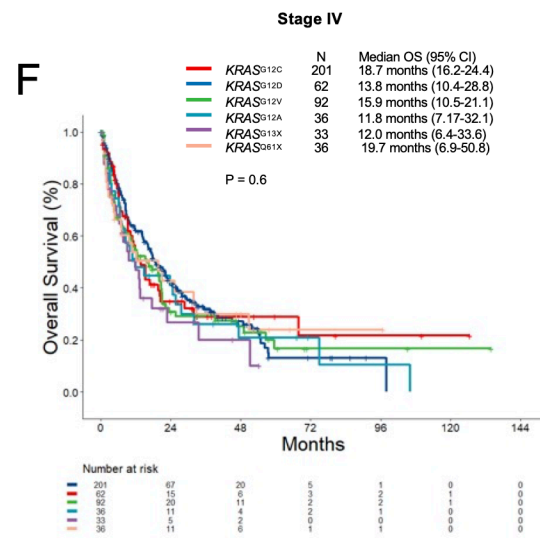
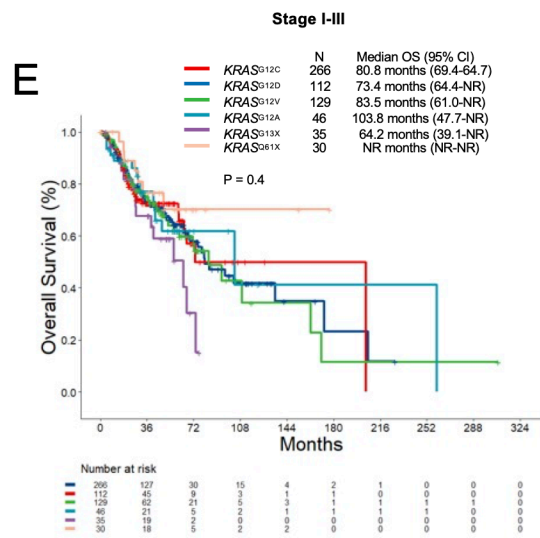
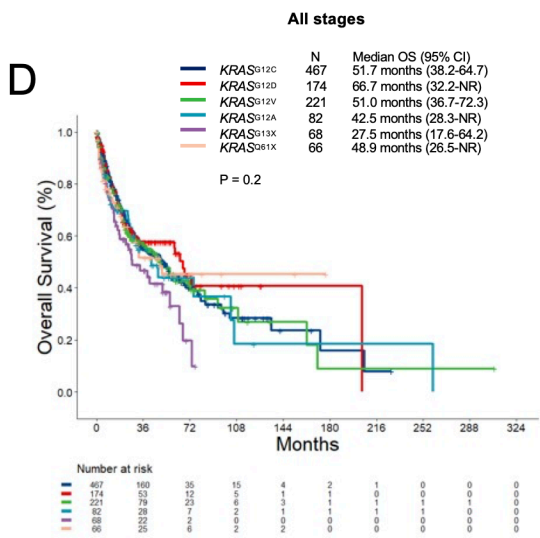
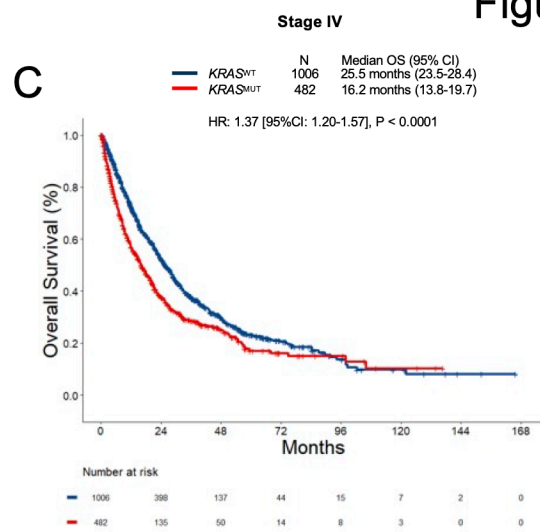
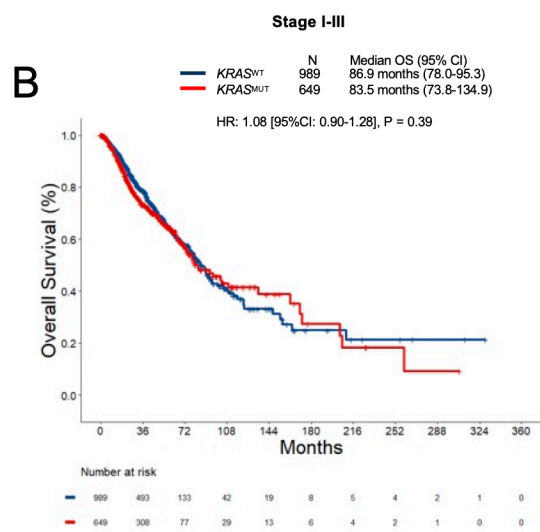
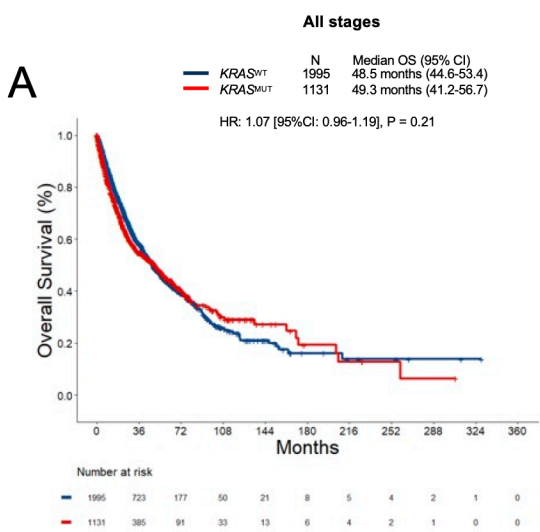


Figure 5

