Dear Dr. Ridd,

We are glad that both reviewers were enthusiastic about the study we have presented. Reviewer 1 believed it to be a thorough analysis of the genetic interactions of the *KRAS* alleles that is timely and novel. Reviewer 2 noted that our findings are likely important for understanding *KRAS*-driven cancers. We believe the analysis has been enhanced by pursuing the insightful questions raised by both reviewers. Below, we have provided both a summary of our responses for each reviewer and a detailed response to each comment from the reviewers (annotated with the locations in the manuscript where the relevant changes were made).

Summary

Reviewer #1

The first reviewer requested additional explanation and analysis of the associations between mutational signatures and the *KRAS* alleles. Reviewer 1 was also curious about the interpretation of our results and the characteristics of the tissue-of-origin of the tumors.

We addressed these concerns as follows:

* We conducted statistical analyses to test for differences in mutational signature compositions of tumor with different *KRAS* alleles (presented in the new Supplemental Fig. 2) and differences in the probabilities for the mutational signatures to have caused the mutation (presented in the new Supplemental Fig. 3). In addition, we assessed to what extent the mutational signatures can be used to predict which *KRAS* allele a tumor would obtain (presented in the new panel b of Fig. 2).
* In multiple parts of the Results and Discussion, we add further interpretation of our results with regards to the tissue-specificity of *KRAS* mutations, citing several experimental studies on the topic to provide further support.

Reviewer #2

Reviewer 2 expressed concern over the clarity of our goals and statistical methods, namely the potential for a high rate of false positives in the comutation networks. It was also unclear to this reviewer why our comutation analysis of the individual *KRAS* alleles would discover genetic interactions not recorded in previous comutation analyses. Lastly, this reviewer requested that we provide additional details about the mutations in genes found to have these comutation interactions with *KRAS*.

We addressed these concerns as follows:

* We have made an effort to clarify the goals and methods of our study throughout the manuscript. We have specifically provided a detailed explanation of the steps we used to mitigate the reporting of false positive comutation interactions and have added an additional step that filters based on an estimated false discovery rate.
* To emphasize the novelty of an allele-specific analysis, we conducted a non-allele-specific comutation analysis of *KRAS* and compared the results to the allele-specific analysis presented in the original manuscript. Briefly, the results highlight how the allele-specific analysis is crucial for identifying most of the comutation interactions of the uncommon *KRAS* alleles as they are often masked by the more common alleles.
* As requested by the reviewer, we have included further details on the particular mutations of the genes with comutation interactions with the *KRAS* alleles.

Point-by-Point Responses

Reviewer #1

(1) Authors showed that there is no difference in mutation signature patterns comparing tumors with distinct KRAS mutations (Fig 1C), the question is that whether tumors with different mutational signatures have preferred KRAS mutations? Regardless of tumor type, what is the correlation between predicted and actual KRAS mutations in tumors with each mutational signature?

After much internal discussion, we ultimately had three different interpretations of these two insightful questions. Each is addressed separately, below.

The first question – “whether tumors with different mutational signatures have preferred *KRAS* mutations?” – can be understood to be asking if tumor samples grouped by their *KRAS* allele have different levels of each mutational signature. We believe this is addressed by Fig. 1c, and the result is that most mutational signatures do not demonstrate higher levels in tumor samples of specific *KRAS* alleles. Originally, we did not include a statistical test for this assertion, thus we have conducted pairwise Wilcoxon rank-sum tests to determine if there was a difference in mutational signature level between cancer samples with different *KRAS* alleles. Our original assertion was correct, but we are now able to specifically indicate which mutational signatures stray from the general conclusion (Supplementary Fig 2).

Another interpretation of the first question is that the reviewer is interested in whether certain mutational signatures have a stronger preference for inducing specific *KRAS* mutations. We believe that this is already addressed by Fig. 1d, which presents the average probability that the observed *KRAS* allele of a tumor was caused by each mutational signature. From this, there are clear associations, several of which are highlighted in the text. For example, the probability for SBS4 to have induced G12A/C/V mutations is much higher compared to G12D mutations in LUAD. Originally, we did not include a statistical test for this assertion, thus, we have conducted pairwise Wilcoxon rank-sum tests to determine if there were differences in the probabilities of alleles to have been caused by each mutational signature. Our original assertion was correct, but we are now able to specifically indicate which mutational signatures stray from the general conclusion (Supplementary Fig 3).

The third interpretation we had of this comment pertained to the second question: “Regardless of tumor type, what is the correlation between predicted and actual *KRAS* mutations in tumors with each mutational signature?”. To begin, it should be noted that multiple mutational signatures are detected in most tumor samples (Supplementary Fig. 1a), thus we were unable to perform an analysis “in tumors with each mutational signature.” In addition, we were unsure whether grouping tumors of different origins would improve our understanding of the results, particularly because any statistic calculated per *KRAS* allele aggregated across cancer types would be unbalanced (e.g. there are far more *KRAS* G12R mutants in PAAD than all of the other cancers, combined).

With these caveats, we otherwise understood the second question to be asking whether if, on a per-tumor-sample basis, there is an association between the probability of each *KRAS* allele (estimated from the mutations found in the tumor sample) and the observed *KRAS* allele (the mutation actually acquired by the tumor). To address this, we analyzed the probabilities of the *KRAS* alleles in each tumor sample via two methods: 1) comparing the probability of obtaining a certain *KRAS* allele between tumor samples that obtained the specific *KRAS* allele and other tumor samples and 2) determining the extent to which the probabilities were predictive of the obtained *KRAS* allele. For most *KRAS* alleles, there was no difference between the probability of obtaining a specific *KRAS* mutation in tumor samples observed to have the allele compared to tumor samples with a different *KRAS* allele (Fig. 2b). The two instances where this was not the case were with G12V in COAD and G12C in LUAD, where each *KRAS* allele had a greater probability of occurring in tumor samples that actually obtained the mutation compared to other tumor samples. Regarding the second analysis, we measured what fraction of tumor samples with a *KRAS* mutation had the observed allele predicted as the most probable (“sensitivity” or “true positive rate”) (Fig. 2c). For comparison, we calculated the same statistic for tumor samples without the given *KRAS* allele (“false positive rate”). To clarify with an example, we compared the fraction of PAAD *KRAS* G12D samples with the G12D allele as the most likely *KRAS* mutation to the fraction of other PAAD tumor samples with G12D predicted as the most likely mutation. The results indicated that the probabilities of the *KRAS* alleles as estimated from the exome/genome mutations were poor predictors of what allele would be obtained; only G12C in LUAD was correctly predicted in over 40% of the LUAD *KRAS* G12C tumor samples. Otherwise, the *KRAS* alleles were correctly predicted at very low rates, and there was little difference between the true positive and false positive rates in most cases. More discussion of the results are included in the manuscript along with a detailed update of the Methods section.

(2) Figure 2 shows the predicted frequencies of KRAS alleles are different from observed frequency without a convincing explanation – please discuss/explain in some detail.

The additions made to address the previous question (1) provide additional explanation of this result. Briefly, the new analysis demonstrates that, on average, each tumor sample was equally likely to obtain each *KRAS* allele compared to other samples of the same cancer type. Taken together with the results of Fig. 2a (previously the only panel of Fig. 2), this indicates that other factors determine the distribution of *KRAS* alleles. We believe these other factors are primarily the interactions between the distinct biological properties of each mutant *KRAS* and the pre-existing signaling context of the tissue-of-origin. In other words, some *KRAS* alleles are stronger cancer drivers than others in different tissues and this, along with the active mutagenic processes, determines the frequency at which the *KRAS* alleles are observed in each cancer.

(3) Please detail how the probability of each mutational signature to have caused the KRAS mutation in a tumor sample is calculated. The statistical analysis for the association between KRAS mutations and mutational signatures needs to be provided.

The probability of each mutational signature to have caused the *KRAS* mutation in a tumor sample is explained in the Methods section “Probability of *KRAS* mutations from mutational signatures.” Some changes to this explanation have been made to clarify the method, and the revised version is copied below:

For each sample harboring a *KRAS* allele, the probability of each mutational signature to have caused the mutation was calculated by considering the weight of the base change among the 96 possibilities and the relative contribution of the signature to the mutations in the sample. Thus, the probability of a tumor sample to have acquired the *KRAS* mutation from signature of all signatures can be calculated using Eq. 1.

# where is the contribution of signature in sample and is the weight of mutation in signature . The probability is normalized to sum to 1 by dividing by the probability of getting the observed KRAS mutation from any of the signatures.

# The probability of a mutational signature to have caused a *KRAS* mutation was compared between two groups of tumor samples separated by their observed *KRAS* allele using a Wilcoxon rank-sum test. The p-values were adjusted for multiple hypothesis testing using the Benjamini-Hochberg method.

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As stated in the response to question (1), we conducted pairwise Wilcoxon rank-sum tests to determine if there were differences in the probabilities of alleles to have been caused by each mutational signature.

(4) Is there additional evidence that supports the supposition that “The alleles never or rarely found in each cancer were predicted to occur at frequencies ranging from 1.5% (for Q61L in PAAD) to 10.5% (for Q61K in LUAD), indicating that these alleles are not rare because their causative mutations do not occur, but instead because of weak oncogenic fitness in the tissue.”

As these are rare *KRAS* mutations in the respective cancers, their oncogenic fitness has not been experimentally tested as thoroughly as the more common alleles.

We do specifically discuss the instance of *KRAS* A146T in PAAD, a very rare allele though predicted to comprise 9% of *KRAS* mutations, as we have experimental evidence of its weak fitness in this context (Poulin *et al*., 2019). In addition, Zafra *et al.*, recently demonstrated multiple tissue-specific responses by expressing *KRAS* G12R in the colon and *KRAS* G13D in the pancreas. For instance, the expression of *KRAS* G12R in the colon was insufficient to induce the hyperplasia that is regularly observed with the more common alleles.

With specific regards to Q61 mutations, there is evidence that, provided sufficient mutagenic pressure, *KRAS* Q61L mutations can induce tumorigenesis in the lungs (Li *et al.*, 2020). In Supplementary Fig. 2, we indicate that the prediction of the frequency of Q61L is very close to the actual frequency, supporting this experimental conclusion.

(5) When performing comutation analysis, do these mutations exist in a clonal population versus polyclonal populations?

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This is an interesting question that we had originally wanted to address in this study. Nevertheless, we were unable to collect and/or calculate the variant allele fraction (VAF) data for every mutation, preventing a comprehensive analysis. As discussed in the manuscript, MM is known to be frequently multi-clonal. We presumed that not accounting for this would lead to a high-rate of false positives in the comutation analysis. Therefore, we focused on genes previously known to drive MM to avoid highlighting false positive interactions. For COAD, LUAD, and PAAD, it should be noted that almost all of the *KRAS* mutations are clonal, thus all other mutation events would be in tumor cells containing the *KRAS* mutation.

(6) Is there any genetic/genomic evidence, such as mutual exclusivity, for these possible ‘synthetic lethal’ genes with KRAS mutations?

Interestingly, there was no overlap between the genes with allele-specific reduced comutation interactions and those with increased genetic dependency. We believe this to be due to two main factors. The first is that the effects of a gene not being mutated is, in most cases, distinct from complete bi-alleleic loss of the gene. Thus, a CRISPR-Cas9 knockout screen does not mimic the cellular effect of mutual exclusive mutations.

The second main factor is that we do not account for gene copy number in the comutation analysis, though many collateral lethal interactions have been attributed to the loss of a single allele (often termed “*c*opy number alterations *y*ielding *c*ancer *l*iabilities *o*wing to *p*artial loss” or “CYCLOPS”; Nijhawan *et al.*, 2012, Muller *et al.*, 2015). Therefore, our comutation analysis would not identify many of the collateral lethality effects that would likely be identified by a genetic screen. This is a limitation of the study.

(7) The analysis and discussion of the relationship between tissue characteristics and specific KRAS mutations are weak and should be strengthened. For example, why does MM have more KRAS Q61H than other cancer types?

This is a very important open question in the field that we believe our paper contributes towards answering, though is insufficient to answer fully. Our results indicate that the distribution of frequencies of the *KRAS* alleles observed in these cancers cannot be solely attributed to passive mutational processes. Instead, we posit that the distinct biological properties of the different *KRAS* mutations contribute to their tissue-specific frequencies. This is supported by how the alleles have distinct tissue-specific genetic interactions. However, the precise biological reasons for the tissue-specificity of *KRAS* mutations has yet to be determined. Thus far, several studies from our lab and others have experimentally examined how some alleles interact with specific tissues (for example: Poulin *et al*., 2019, Hobbs *et al.*, 2019, and Zafra *et al.*, 2020). We believe understanding these phenomena is essential to fully describing *KRAS* driven cancers and are continuing to pursue it from various perspectives.

(8) The observed frequency of some KRAS alleles was greater than predicted perhaps due to positive selection, but quite a few KRAS alleles occurred at significantly lower frequency than predicted in some cancers, possibly due to weak oncogenic fitness in the specific tissue. This issuea needs to be explained and discussed.

We agree that this is an important point to address. It is discussed in the 3rd paragraph of the Results section “The frequency of most *KRAS* alleles cannot be solely attributed to the prevalence of detected mutagens” with specific reference to the high expected frequency of A146T mutations in PAAD and its experimentally-demonstrated poor oncogenic fitness.

(9) The study provided many statistically significant findings, but did not link these findings to cancer- and allele- specific differences in drug response and clinical outcome. For example, the background conveys that COAD tumors with a KRAS G13D allele are sensitive to anti-EGFR therapies; and advanced PAAD with KRAS G12D allele is associated with reduced overall survival. It would be helpful to discuss whether allele-specific comutations or differentially dependent cellular processes or other oncogenes could explain/contribute to such observations.

Additional discussion of this topic has been added to the Discussion. We believe this is a very important topic and was the reason for the paper’s final analysis “An integrated analysis of allele-specific comutation and genetic dependencies.” The purpose of this analysis was to test whether *KRAS* allele-specific dependency interactions could alternatively be explained by mutations to genes with comutation interactions with the *KRAS* allele. This was inspired by the possibility that distinctions between *KRAS* alleles identified from ad hoc analyses of clinical data could actually be driven by mutations to other genes that happen to have comutation patterns with the *KRAS* alleles.

In addition, we have tried to statistically analyze the associations between comutation events and patient outcome, though we were limited by data availability. High-quality clinical data is relatively uncommon, thus, when grouping the patients by the *KRAS* mutation of their tumor and the mutation status of comutating genes, the sub-groups become too small to provide sufficient statistical power to identify distinctions in patient outcome.

(10) The github code is not available with the link provided. ( <https://github.com/jhrcook/comutation> ). There are no supplementary tables 2-9.

We apologize for the oversight on the availability of the code; the repository has been made public. For the supplementary tables, it seems that the Excel file was turned into a PDF at some point in the submission process. The entire Excel file should now be available to the reviewers.

Reviewer #2

(1) This is an intriguing study with possible implications for KRAS driven cancers. However, the authors need to make their methods and goals far more transparent and easier for the reader to understand. Moreover, it wasn’t clear if all the statistical tests (such as the comutation studies) had been adjusted for multiple testing.

Per the reviewer’s recommendation, we have improved the clarity of the methods and goals in the manuscript.

With specific regards to the statistical analyses of the comutation studies, we did not filter the comutation interactions based on FDR-adjusted p-values because we found these methods too strict for our purpose - removing all but the strongest interactions (e.g. the reduced comutation interactions with *BRAF* or increased comutation interactions with *APC* in COAD). Instead, we opted to use a relatively strict p-value cutoff (p-value < 0.01) and additional thresholds on other properties of the comutation interactions (such as a lower bound on the number of comutation events) to remove likely false positives. We believe this thresholding process eliminated many false positives while still providing a meaningful list of comutation interactions for further *in vitro* study. Recognizing these limitations, we chose to focus on genes known to be related to cancer or *KRAS* signaling and the results of a functional enrichment analysis to specifically highlight the comutation interactions least likely to be false positives. In this vain, we have applied an additional filter to the interactions with genes previously associated with cancer or *KRAS* signaling (those presented in Fig. 3b, Supplementary Fig. 6b and Supplementary Fig. 8b). These sets of interactions were further filtered to fall below an FDR of 0.25 that was estimated using the Benjamini-Hochberg method.

The adjustments for multiple hypothesis testing for the remaining analyses are specifically indicated in their respective Methods sections.

(2) Comutation networks to this reviewer are puzzling. One would expect that comutated genes would have been identified as genetic drivers already. The fact that many of them have not suggests that results could be spurious. Could the authors please address this issue.

The hypothesis behind this allele-specific comutation analysis is that some mutations only contribute to cancer within a specific cellular context, in this case, the unique signaling characteristics of a specific *KRAS* allele. These genes may not have been previously documented as cancer drivers as they do not behave as such on their own. Instead, they only promote cancer when accompanied by additional specific signaling perturbations.

Previous comutation studies that group all *KRAS* mutations into a single category do so under the assumption that they are identical. However, if the *KRAS* alleles are in fact distinct, then allele-specific interactions are likely to be missed, particularly for the less common alleles. Just as with any comutation analysis, interactions can be spurious, though, as explained in the previous question, we have used several methods to highlight interactions most likely to be real.

To demonstrate how the results of an allele-specific and non-allele-specific comutation analysis differ, we conducted the same statistical tests for comutation interactions currently used in the study, but now treating all *KRAS* mutations as a single group. The identified interactions were compared to those from the allele-specific analysis and are now present in Supplementary Fig. 5.

In COAD, LUAD, and PAAD the sets of genes found to have increased or reduced comutation interactions were substantially different between the two analyses (Supplementary Fig. 5a, c, and g). In MM, the results are quite similar (Supplementary Fig. 5e), though there were only a few interactions identified in total and there is still the factor of possible polyclonality of these tumors (as discussed in the manuscript).

For COAD and LUAD, the interactions found from the two different analyses (allele-specific and non-allele-specific) had both considerable overlap and distinction. For example, the non-allele-specific *KRAS* comutation analysis in COAD identified 105 reduced comutation interactions, only 35 of which were also identified in the allele-specific analysis (Supplementary Fig. 5a). On the other hand, 28 novel reduced comutation interactions were only identified when the *KRAS* alleles were considered individually. The fact that 70 genes were identified only by the non-allele-specific analysis indicates that there may be some genes that comutate with several, but not all, *KRAS* alleles, and the allele-specific analysis was under-powered to identify them. Overall, the results of comparing the allele-specific and non-allele-specific analyses demonstrate that they address similar, yet distinct, biological relationships.

In PAAD, the value of an allele-specific analysis is highlighted because upwards of 90% of the tumors have a *KRAS* mutation. Therefore, a non-allele-specific analysis identified 5 reduced comutation interactions and 6 increased comutation interactions (Supplementary Fig. 5g). Conversely, the allele-specific analysis identified far more interactions, including multiple genes that simultaneously have increased comutation with some *KRAS* alleles and reduced comutation with others (Supplementary Fig. 8c).

Due to limitations on the length of the text, we were unable to include this complete explanation in the manuscript. However, we have highlighted the main conclusions in the figure’s legend.

(3) Page 5: State which KRAS alleles were correlated with microsatellite instability.

The following was appended to the end of the Results section “*KRAS* alleles are non-uniformly distributed across cancers”:

Because around 17% of COAD tumors were hypermutants, we used a one-sided Fisher’s exact test to determine if any *KRAS* alleles were enriched in these tumors. Overall, hypermutant samples were more likely to be WT *KRAS* (odds ratio = 1.2, FDR-adjusted p-value < 0.05), though *KRAS* Q61K was correlated with hypermutant samples (odds ratio = 6.3, FDR-adjusted p-value < 0.001). It should be noted that Q61K is a very rare allele in COAD, found in 0.47% of the tumor samples.

(4) Page 5, last paragraph: Reword first sentence – there are better ways of saying this.

The sentence, “Each mutational process is not equally likely to cause each *KRAS* allele,” has been changed to, “Each mutational process has a different propensity to induce each *KRAS* allele.”

(5) Page 7, para, line 6: Missing words here: but “had an” actual….

The sentence, “... Q61H, which was dramatically underestimated with a predicted frequency of 15.0% but actual frequency of 35.7% of KRAS mutations,” was changed to, “... Q61H, which was dramatically underestimated with a predicted frequency of 15.0% but **an** actual frequency of 35.7% of KRAS mutations.

(6) Page 7, para 2, line 2: Presumably the authors are referring to KRAS alleles here – if so please insert.

The sentence, “correlations between the observed and predicted allele frequencies for each cancer…” was changed to “correlations between the observed and predicted ***KRAS*** allele frequencies for each cancer…”

(7) Page 8, last para, line 3: Don’t the authors mean known oncogenes and tumor suppressor genes? Similarly p 9, last para – TP53 is a tumor suppressor etc.

The sentence, “... or are known oncogenes…” was changed to “... or are known oncogenes **or tumor suppressor genes**…” The text was also changed in similar contexts elsewhere in the manuscript.

(8) It would have been helpful for the authors to describe the actual mutations in genes where there was significant comutation (or lack of) with the KRAS alleles. For example in the case of TP53, was this affected by whether the mutation was truncating, or affected protein interaction? Similarly for the genetic dependencies from the CRISPR screens, what are the actual mutations in the genes identified (shown in Figure 4d?).

Brief descriptions of mutations to genes found to have comutation interactions with *KRAS* alleles have been included where relevant in the Results section “The *KRAS* alleles have distinct comutation networks.” In most cases, these mutations were those commonly found of the known cancer-associated genes. However, there were some notable trends such as the uniquely high rate of comutation between *KRAS* G12V and the *TCF7L2* R488C mutation.

We inspected the mutations to the genes with opposing comutation interactions with multiple *KRAS* alleles highlighted in Supplementary Fig. 5 (*TP53*, *RNF43*, *MAP2K4*, *RBM10*), though found no notable trends. The types of mutations were briefly described in the text.

To further describe the interactions with known oncogenes, analyses of the comutation between the alleles of oncogenes and those of *KRAS* have been conducted. No patterns of *KRAS* alleles demonstrating differential preference for specific alleles of other oncogenes was uncovered. For instance, in the analysis of *PIK3CA* in COAD, there was a significant association of *KRAS* G12C, G12V, and G13D with *PIK3CA* Q546K and E545K for increased rates of comutation, though there were no *KRAS* alleles with reduced comutation interactions with any *PIK3CA* alleles.

A similar analysis was conducted for the comutation between *KRAS* alleles and individual protein domains of tumor suppressor genes (TSG). As above, no new interactions were identified where *KRAS* alleles demonstrated distinct patterns of comutation with particular domains of TSG.

Details of the mutation to *TP53* in the COAD cell lines and *SMAD4* in the PAAD cell lines were added to the Results section “An integrated analysis of allele-specific comutation and genetic dependencies.”:

“Most of the *TP53* mutations were located in the DNA binding domain, two of which were nonsense mutations. Of the other mutations, two were at splice-sites, one was in the nuclear localization signaling domain, and two more were either nonsense or frameshift mutations in the N-terminal domain. All were either predicted to be deleterious or are at hotspots previously identified by TCGA or COSMIC… All of the *SMAD4* mutations were at known COSMIC hotspots. All but two were frameshift or nonsense mutations.”

(9) The authors report a high rate of co-mutation between KRAS Q61H alleles and NRAS in MM. Describe which allele/s of NRAS these were. Has this been reported before for MM?

The concomitant mutations were predominantly at *NRAS* Q61 though there was no detectable pattern of comutation between specific *KRAS* and *NRAS* alleles (possibly due to the low power of the analysis caused by further subdividing the groups of tumors). This information has been added to the text in the 2nd-to-last paragraph of the Results section “The *KRAS* alleles have distinct comutation networks.”

The co-occurence of *KRAS* and *NRAS* mutations in multiple myeloma have been reported previously including by Bolli *et al*. in “Heterogeneity of genomic evolution and mutational profiles in multiple myeloma” (2014, PMID: 24429703)