RAS mutation patterns arise from tissue-specific responses to distinct oncogenic signaling

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Abstract

Despite multiple possible oncogenic mutations in the proto-oncogene *KRAS*, unique subsets of these mutations are detected in different cancer types. As *KRAS* mutations occur early, if not being initiating, these mutational biases are ostensibly a product of how normal cells respond to the encoded oncoprotein. Oncogenic mutations can impact not only the level of active oncoprotein, but also engagement with effectors and other proteins. To separate these two effects, we generated four novel inducible *Kras* alleles encoded by the biochemically distinct mutations G12D versus Q61R encoded by native (nat) rare versus common (com) codons to produce either low or high protein levels. Each allele induced a distinct transcriptional response in normal cells. At one end of the spectrum, the *Kras^{natG12D}* allele induced transcriptional hallmarks suggestive of an expansion of multipotent cells, while at the other end, the *Kras^{comQ61R}* allele exhibited all the hallmarks of oncogenic stress and inflammation. Further, this dramatic difference in the transcriptomes of normal cells appears to be a product of signaling differences due to increased protein expression as well as the specific mutation. To determine the impact of these distinct responses on RAS mutational patterning *in vivo*, all four alleles were globally activated, revealing that hematolymphopoietic lesions were sensitive to the level of active oncoprotein, squamous tumors were sensitive to the G12D mutant, while carcinomas were sensitive to both these features. Thus, we identify how specific *KRAS* mutations uniquely signal to promote the conversion of normal hematopoietic, epithelial, or squamous cells towards a tumorigenic state.

Introduction

Why specific driver mutations track with different cancers is unknown, yet holds the key to understanding the origins of cancer, with implications for early detection and prevention. This is particularly well illustrated with the small GTPase KRAS. Single point mutations at one of three hotspot positions (G12, G13, and Q61) result in six possible substitutions that inhibit the intrinsic or extrinsic GTPase activity of the protein, rendering KRAS constitutively GTP-bound and active, which is well known to be oncogenic (*Simanshu et al., 2017*). Despite 18 possible oncogenic mutations, specific subsets of these mutations tend to be found in specific cancer types. For example, G12C is the most common KRAS mutation in non-small cell lung cancer while it is Q61H in plasma cell myeloma (*Li et al., 2018; Prior et al., 2020*). Mice similarly exhibit a bias of specific *Kras* mutations towards different cancer types, as different mutant *Kras* alleles have different tumorigenic potential when activated in different tissues (*Li et al., 2018; Poulin et al., 2019; Winters et al., 2017; Wong et al., 2020; Zafra et al., 2020*). While this tissue ‘tropism’ of cancers towards specific KRAS mutations has been appreciated for decades (*Bos, 1989*), the underlying mechanism is unclear.

Variation in the ability of specific KRAS mutants to be tumorigenic (or not) in different tissues ostensibly results from differences in oncogenic signaling between mutants. Generally speaking, oncogenic signaling is a product of the amplitude of the signal (quantitative signaling) and/or the effector pathway engaged (qualitative signaling). In terms of quantitative signaling, different mutations can exhibit different degrees of activation (GTP-loading) and/or different sensitivities to positive (Ras GTP exchange factors, RASGEFs) or negative (RAS GTPase activating proteins, RASGAPs) regulators (*Gebregiworgis et al., 2021; Lu et al., 2016; Muñoz-Maldonado et al., 2019; Simanshu et al., 2017*). Various methods to manipulate quantitative *Kras* signaling, such as through modulating recombination rates (*Singh et al., 2020*), homozygous expression of the mutant allele (*Burgess et al., 2017*), changing codon usage to increase translation (*Pershing et al., 2015*), additional pharmacologic activation of the mitogen activated protein kinase (MAPK) pathway (*Cicchini et al., 2017*), and so forth (*Li et al., 2018*), all affect tumorigenesis. On the other hand, perhaps one of the best examples of qualitative differences in KRAS signaling is the G12R mutant. Unlike the more canonical G12D mutation, the G12R mutant exhibits reduced

binding the RAS effector PI3K α and PI3K/AKT signaling (*Hobbs et al., 2020; Zafra et al., 2020*). Activating an inducible *Kras*^{G12R} allele in the pancreas led to early premalignant lesions compared to the much more tumorigenic *Kras*^{G12D} allele (*Zafra et al., 2020*). Despite an appreciation that different KRAS mutations can manifest in quantitative or qualitative signaling differences, how these two signaling outputs impact the mutational patterns of this oncogene was unclear.

As KRAS mutations are often initiating, being sufficient to induce tumorigenesis in mice and truncal in many human cancers, the bias of specific mutations towards distinct cancers may arise from tissue-specific responses of normal cells to quantitative and qualitative features of KRAS signaling (*Li et al., 2018*). Determining the immediate response of normal cells to different *Kras* mutations *in vivo* thus holds the key to understanding the mutational patterning of this oncogene. Identifying a point mutation arising in *KRAS* from the cell-of-origin prior to becoming a tumor is challenging in humans. Mice, on the other hand, provide an ideal model system to experimentally explore this phenomenon, as the point of tumor initiation can be precisely defined using inducible oncogenic *Kras* alleles. To thus determine why specific KRAS mutations have such a strong bias for different cancer types we created four novel inducible murine *Kras* alleles designed with very different oncogenic mutations that were expressed at either low or high levels. In this way, tissues sensitive to quantitative signaling would develop tumors dependent on the activation status of the *Kras* oncoprotein, whereas those sensitive to qualitative signaling would develop tumors dependent upon the mutation type.

We chose two completely different oncogenic mutations for these experiments, namely G12D and Q61R. G12D places a negatively charged head group into the catalytic cleft of RAS and blocks extrinsic (RASGAP-mediated) GTPase activity (*Parker et al., 2018*). On the other hand, Q61R replaces the catalytic amino acid with one that has a positively charged headgroup, disrupting the position of the active site water molecules necessary for intrinsic GTP hydrolysis (*Buhrman et al., 2010*). Q61 is also essential for extrinsic GTP hydrolysis, as it stabilizes the transition state via hydrogen bonds to the γ -phosphate and nucleophilic water while providing another hydrogen bond to the GAP arginine finger (*Grigorenko et al., 2007; Kotting et al., 2008; Rabara et al., 2019; Scheffzek et al., 1997*). Comparing these two mutants directly reveals Q61R to have significantly lower GTP exchange and GTP hydrolysis rates than G12D (*Burd et al., 2014*;

Rabara et al., 2019), akin to other substitutions at these two positions (*Gebregiworgis et al., 2021; Smith et al., 2013*). In those few cases in which the tumorigenic potential of the G12D and Q61R mutants have been directly compared in mice, tissue specific expression of *Nras*^{G12D} or *Kras*^{G12D} was less potent than their Q61R counterparts at inducing melanoma (*Burd et al., 2014*) or myeloproliferative neoplasm (*Kong et al., 2016*), respectively.

To parse out the contribution of quantitative oncogenic signaling, the *Kras* alleles encoding these two different mutations were expressed at different levels by manipulating their codon usage. Namely, the first three coding exons were fused and encoded by either their native rare codons, which is known to retard protein translation, or common codons to increase translation (*Lampson et al., 2013; Pershing et al., 2015*). We chose the novel approach of altering mammalian codon usage to modulate protein expression (*Pershing et al., 2015*), as no additional elements are required to change protein levels, providing a simple, reproducible, and uniform way of precisely controlling *Kras* levels in mice and derived cell lines.

These four alleles were activated and immediately thereafter the transcriptome of normal cells was determined, which revealed that each allele induced a specific transcriptional response in normal cells. Increased expression shifted the transcriptional hallmarks consistent with an expansion of multipotent cells to that of oncogenic stress and inflammation. Changing the mutation shifted the hallmark of estrogen response in the G12D mutant to that of the p53 pathway and DNA repair in the Q61R mutant. To determine how these two types of signaling contribute to RAS mutational patterning *in vivo*, all four alleles were globally activated, revealing that hematolymphopoietic lesions were sensitive to the level of active oncoprotein, squamous tumors were preferentially sensitive to the G12D mutant, while carcinomas tended to be sensitive to both these changes. Thus, we identify how specific KRAS mutations uniquely signal to promote the conversion of normal hematopoietic, epithelial, or squamous cells towards a tumorigenic state.

Results

A panel of *Kras* alleles designed to separate the effects of a mutation from the activity of the oncoprotein

To elucidate how specific cancers are driven by specific KRAS mutations, we reasoned that the contribution of quantitative signaling of the oncoprotein could be parsed out by simply changing the amount of protein made, while the contribution of qualitative signaling could be parsed out by using two different oncogenic mutants. To this end, we created the four novel *LSL-Kras^{natG12D}*, *LSL-Kras^{natQ61R}*, *LSL-Kras^{comG12D}*, and *LSL-Kras^{comQ61R}* alleles (**Figure 1A**) in which an LSL transcriptional/translational repressor sequence (STOP) flanked by *loxP* sites (**Jackson et al., 2001**) was engineered into the first intron of *Kras* after the non-coding exon 0 to provide temporal and special control of gene expression. This was followed by a fusion of coding exons 1 to 3 encoded by either their native (*nat*) rare codons, which are known to retard protein translation, or 93 of these rare codons converted to their common (*com*) counterparts to increase protein expression (**Figure 1—figure supplement 1,2**), and either a G12D or Q61R mutation. As noted above, each of these mutants alters RAS activity in a biochemically different manner (**Muñoz-Maldonado et al., 2019; Simanshu et al., 2017**), with Q61R reported to yield higher levels of active (GTP-bound) Ras (**Burd et al., 2014; Kong et al., 2016; Pershing et al., 2015**). This was followed by the next intron containing an FRT-NEO-FRT cassette for ES selection, which was excised via Flp-mediated recombination after which the Flp transgene was outbred. Finally, the remainder of the gene was left intact so as to generate the two *Kras4a* and *Kras4b* isoforms, as both contribute to tumorigenesis (**To et al., 2008**), potentially through unique protein interactions (**Amendola et al., 2019**).

All four alleles were successfully recombined by Cre recombinase, as confirmed with two separate mouse embryonic fibroblast (MEF) cultures derived from each genotype (**Figure 1B, and Figure 1—source data 1**). Based on common codons increasing protein expression and Q61R leading to higher levels of GTP-bound RAS, all four alleles displayed the expected gradual increase the level of active *Kras*. Namely, we confirm a stepwise increase in ectopic GTP-bound *Kras* in the ascending order of *Kras^{natG12D}* < *Kras^{natQ61R}* < *Kras^{comG12D}* < *Kras^{comQ61R}* by both immunocapture

and ELISA-based assays (**Figure 1C** and **Figure 1—figure supplement 3**). Finally, we confirm that these four alleles exhibit the expected increase in oncogenic signaling *in vivo*. Namely, we crossed the *LSL-Kras^{natG12D}/+*, *LSL-Kras^{natQ61R}/+*, *LSL-Kras^{comG12D}/+*, and *LSL-Kras^{comQ61R}/+* genotypes into a *Rosa26-CreERT2* background, which expresses a tamoxifen-inducible Cre from the endogenous *Rosa26* promoter that is active in a broad spectrum of tissues (**Ventura et al., 2007**). Two adult mice from each of the four derived cohorts, as well as the control strain (*Rosa26-CreERT2/+*), were injected with tamoxifen and seven days later humanely euthanized, their lungs removed, RNA isolated, and the level of mRNA encoded by RAS target genes determined by qRT-PCR (**Figure 1—figure supplement 4A**). This revealed the expected increase in three of the target genes by the *LSL-Kras* alleles when encoded with common codons, which was further increased in the Q61R-mutant background (**Figure 1—figure supplement 4B,C**). Thus, the four novel *LSL-Kras* alleles function as designed.

The effect of codon usage and mutation type on Kras biological activity

As the four alleles exhibited the expected biochemical and signaling properties of the encoded proteins, we next confirmed that these alleles were proportionally tumorigenic in a side-by-side comparison in the same organ. Each of these four alleles was specifically activated by tamoxifen injection to induce expression of Cre recombinase in the lung, after which every month thereafter for six months, five mice from each of the four cohorts were humanely euthanized (**Figure 2—figure supplement 1A**). The lungs from all mice were visually analyzed for the presence of surface pulmonary tumors (**Figure 2—figure supplement 1B**), and in addition, two H&E-stained sections from pairs of mice were assayed for the presence and type of pulmonary tumors by a veterinarian pathologist blinded to the genotype (**Figure 2A**). This revealed a stepwise increase in early onset and tumor burden of pulmonary lesions in lock step with the increased biochemical activity and signaling amplitude of the oncoproteins in the ascending order of *Kras^{natG12D}* < *Kras^{natQ61R}* < *Kras^{comG12D}* < *Kras^{comQ61R}* (**Figure 2A,B**). These tumorigenic phenotypes were the result of activating the *LSL-Kras* alleles in the lungs, as five control *Kras^{+/+}* mice injected with tamoxifen failed to develop tumors after 13 months, twice the length of the study (**Figure 2—figure supplement 1C,D**). Finally, we find preliminary evidence that the G12D-mutant alleles

preferentially led to atypical alveolar hyperplasia (AAH), whereas bronchiolar hyperplasia/dysplasia (BH) lesions were more prevalent with the Q61R-mutant alleles (**Figure 2—figure supplement 1E,F**). We conclude that this allelic set exhibits the expected increase in tumorigenic potential consistent with the activity and signaling of the encoded oncoproteins, but also exhibited evidence of mutation-specific effects on tumorigenesis.

The response of normal cells to different *Kras* codon usage or mutations

As different tumor types and grades induced by each of these four oncogenic *LSL-Kras* alleles are presumably a product of tumor initiation, the key to understanding these effects must lie in how normal cells respond to these different oncoproteins. As the lung was sensitive to both the type of oncogenic mutation and the codon usage of the activated *Kras* allele, we compared the immediate transcriptional response to activation of each allele in this organ in the aforementioned *Rosa26-CreERT2/+* background. Cohorts of three adult male and female mice from each of the four cohorts were injected with tamoxifen, seven days later the animals were humanely euthanized, their lungs removed, and RNA isolated for bulk transcriptome sequencing (**Figure 3—figure supplement 1A** and **GSE181628**).

This revealed that increasing *Kras* activity (through the combination of mutation type and codon usage) progressively moved the transcriptional signature away from one more similar to control tissue (*Kras^{natG12D}*) to one much more complex and with features consistent with high oncogenic activity (*Kras^{comQ61R}*) (**Figure 3A** and **Figure 3—figure supplement 2-5**). Focusing on the two extreme transcriptional responses, we repeated this analysis to ensure reproducibility, finding that activation of the *Kras^{natG12D}* allele led to transcriptional signatures suggestive of the expansion of multipotent cells (**Figure 3—figure supplement 1B,C** and **GSE181627**). Namely, GSEA hallmarks indicative of Epithelial Mesenchymal Transition (EMT, TGF β Signaling, Wnt/ β catenin Signaling, Notch Signaling, Apical Junction, Apical Surface, and Estrogen Response Early) and multiple cell lineages (Adipogenesis, Myogenesis, Hedgehog Signaling, and Pancreas β Cells). Conversely, activation of the *Kras^{comQ61R}* allele had all features of a potent oncogenic signaling leading to oncogenic-induced stress. Namely, GSEA hallmarks indicative of high oncogenic signaling (KRAS Signaling UP) and unrestrained proliferation (MYC Targets V1, E2F

Targets), leading to reactive oxygen species (Oxidative Phosphorylation, Reactive Oxygen Species Pathway) and a DNA damage response (DNA Repair, P53 Pathway, G2M Checkpoint) followed by apoptosis (Apoptosis) and inflammation (Inflammatory Response, IL6 JAK STAT3 Signaling, TNF α Signaling via NF- κ B, Allograft Rejection). STRING analysis of the top ten genes from each of these GSEA hallmarks revealed clear crosstalk between the various signaling pathways, implying a consolidated signaling program by the two different oncoproteins manifests in very different responses by normal cells (**Figure 3B**). We thus suggest that this allelic set moves the response of normal cells from an expansion of multi-potent cells to one of extreme oncogenic signaling and stress.

To extract the effect of quantitative from qualitative signaling in this progression, we first performed principle component analysis on the four transcriptomes, which revealed that both *Kras^{nat}* alleles were distinct from both *Kras^{com}* alleles, while the G12D mutant was most distinct from the Q61R mutant in the *Kras^{com}* background (**Figure 3—figure supplement 1D** and **GSE181628**). Next, we compared the transcriptional responses of both *Kras^{nat}* alleles with both *Kras^{com}* alleles to identify quantitative signaling differences, and compared both *Kras^{G12D}* alleles with both *Kras^{Q61R}* identify qualitative signaling differences. With regards to quantitative signaling, the two *Kras^{com}* alleles induced signaling events related to oncogenic stress, such as an increase in Oxidative Phosphorylation, Glycolysis, Reactive Oxygen Species, MTORC1 signaling, Peroxisome, Xenobiotic Metabolism, and UV Response UP hallmarks and a decrease in UV Response Down, Hedgehog Signaling, and Apical Junction hallmarks. Conversely, the two *Kras^{nat}* alleles decreased KRAS Signaling UP hallmarks (**Figure 3C** and **Figure 3—figure supplement 6**). We interpret this as expression level largely dictating the degree of oncogenic signaling in this allelic set. With regards to qualitative signaling, there were transcriptional signatures preferentially induced by specific mutations that were independent of codon usage. Namely, activation of both G12D-mutant alleles induced the Estrogen Response Late hallmark. On the other hand, we found that activation of the Q61R-mutant alleles induced DNA Repair and P53 Pathway and decreased Interferon Alpha Response hallmarks, suggesting that there was a strong tumor suppressive response uniquely activated by these mutant alleles (**Figure 3D** and **Figure 3—figure supplement 6**).

Finally, we validated the specific signaling responses detected by bulk RNAseq analysis by quantifying four to six marker genes in a subset of selected hallmarks. Namely, two adult mice from each of the four cohorts and control mice were injected with tamoxifen as above, and seven days later the animals were humanely euthanized, their lungs removed, RNA isolated, and the level of select transcripts determined by qRT-PCR. We identified similar expression patterns to the transcriptome signature in the hallmarks of TNF α Signaling via NF κ B and Interferon- γ , which increase with Kras activity (*Figure 3—figure supplement 7A,B*), and EMT and Myogenesis, as these hallmarks were enriched in G12D mutants but depleted in Q61R mutants (*Figure 3—figure supplement 7A,C*).

Thus, in broad terms, we find that increasing Kras activation leads to progressively higher oncogenic signaling, eventually reaching the point of oncogenic stress, with the type of oncogenic mutation further modulating this response. Thus, quantitative signaling differences predominate between these mutants, with evidence of qualitative differences, potentially amplified at higher activation levels.

Tissue sensitivities to different RAS signaling

With the biochemical, biological, and signaling output of each allele now determined, we next addressed the tropism of tissues towards specific oncogenic signaling by determining the tumor landscape upon globally activating each allele. As each of these alleles is expected to have different oncogenic potential, we opted for a moribundity endpoint, as opposed to a fixed endpoint, to identify the tissues most sensitive to tumorigenic conversion in a competition-based approach. To this end, each allele was again activated by tamoxifen using the aforementioned ubiquitous Cre driver *Rosa26-CreERT2*. Mice were then regularly monitored for moribundity endpoints, indicative of ensuing mortality due to cancer, at which point the animals were humanely euthanized (*Figure 4—figure supplement 1*). As a control to rule out a tumor phenotype being a product of variations in gene activation, we validated Cre-mediated recombination between all four alleles across 14 diverse tissues seven days after tamoxifen injection, with only the ovary displaying reduced recombination, and hence was not included in the study (*Figure 4—figure supplement 2*, *Figure 4—source data 1*, and *Supplementary File 1*).

Plotting the percent survival for each of the four genotypes by the Kaplan-Meier approach revealed that the median lifespan of mice progressively increased from 14 days in the case of the activated *Kras*^{comQ61R} allele to 150 days with the *Kras*^{natG12D} allele (**Figure 4A**). Not surprisingly, the number of tumors per animal mirrored these survival differences (**Figure 4—figure supplement 3**). Pairwise comparisons of the survival curves revealed median survivals were statistically different, except between the *Kras*^{natG12D} versus *Kras*^{natQ61R} cohorts (**Figure 4—figure supplement 4** and **Supplementary file 2**). Of note, median survival was significantly decreased in mice with either of the *Kras*^{com} alleles or with either of the Q61R mutants. As both common codons and the Q61R mutation increase Kras activity, tumorigenic potential, and signaling, we suggest that the level of active oncoprotein is the dominant determinant of cancer survival in this model.

To identify the tissue sensitivity to these different alleles, eight different organs were removed from the mice at necropsy and analyzed for pathologic changes as above. While there was much overlap, the prevalence and severity of specific cancer types varied between alleles, arguing that the different alleles can lead to differences in the tumor landscape (**Figure 4B** and **Figure 4—figure supplement 5**). This manifested in four general patterns of tissue sensitivity: hematolymphopoietic neoplasias increased in severity with the level of oncoprotein activation, squamous tumors were preferentially induced by the G12D-mutant alleles, carcinomas exhibited both these features, while many organs were resistant to oncogenic RAS-driven tumorigenesis within the timeframe of the study (**Figure 4A,B**, **Figure 4—figure supplement 5**, and **Supplementary file 3**). Collectively, these observations argue that all tested *LSL-Kras* alleles are oncogenic, with some tissues being sensitive and others resistant, and the response to these four alleles being variable, implying both qualitative and quantitative signaling differences contribute to the bias of tissue-specific oncogenicity of RAS mutations.

A tissue sensitive to quantitative signaling

The incidence of hematolymphopoietic neoplasias increased with the biochemical activity, tumorigenic potential, and signaling amplitude of the oncoproteins. In more detail, the spleen and thymus from eight to ten mice from all four cohorts were removed at necropsy, after which

H&E-stained sections were assayed for the presence and grade of hematolymphopoietic neoplasias as above. Beginning with the least active oncoprotein, most of the *Kras*^{natG12D} mice had no evidence of hematolymphopoietic neoplasms, although some mice had pathologic features consistent with malignant lymphoma. The incidence of malignant lymphoma increased with *Kras*^{natQ61R} allele, and then again with the *Kras*^{comG12D} allele. Pathological analysis also revealed medullary hyperplasia in the thymus, as well as leukemic infiltrates in the kidneys and pancreas of these latter mice. Lastly, *Kras*^{comQ61R} induced severe myeloproliferative disease (MPD) at 100% penetrance, with extensive myeloproliferative infiltrates throughout many tissues (*Figure 4B,C, Figure 4—figure supplement 5, Supplementary files 3,4*, and not shown). While we cannot discount high oncogenic activity leading to a different hematopoietic disease in these later mice, it seems more likely that this incredibly short latency for the onset of severe systemic myeloid neoplasia results in high mortality that prevents development of longer latency tumors such as the development of lymphopoietic neoplasms. Thus, with this proviso, hematolymphopoietic neoplasia are hyper-sensitive to quantitative oncogenic signaling, being induced at the lowest level of active Kras and progressively worsening with increased activity.

Tissues sensitive to qualitative signaling

Proliferative lesions of forestomach and oral squamous epithelium exhibited evidence of qualitative signaling impacting tumorigenesis, namely tumors in these tissues were preferentially induced by the oncogenic G12D mutant of Kras encoded by either native or common codons. In the case of the forestomach tumors, pathological analysis performed as above revealed that the *Kras*^{natG12D} allele induced squamous hyperplasia as well as mild and moderate grades of ‘atypical’ or dysplastic squamous lesions in the forestomach mucosa. Conversely, we did not detect any squamous proliferative changes in the *Kras*^{natQ61R} mice (*Figure 4B,D, Figure 4—figure supplement 5, and Supplementary files 3,4*), despite the previously detected higher GTP-loading, tumorigenic potential, and signaling amplitude of *Kras*^{natQ61R}. The same trend was observed in the *Kras*^{com} background, except the difference was less extreme between the two mutants, and shifted towards more aggressive disease. Specifically, the *Kras*^{comG12D} induced more severe grades of forestomach squamous lesions while *Kras*^{comQ61R} now induced lesions that were of

moderate grades (*Figure 4B,D, Figure 4—figure supplement 5, and Supplementary file 4*). Similar analysis of oral tumors revealed that activating the *Kras*^{natG12D} allele induced minimal to mild grade squamous lesions, while the *Kras*^{natQ61R} allele was not tumorigenic. Again, the *Kras*^{com} background shifted disease to a more aggressive state. Namely, activating the *Kras*^{comG12D} allele induced severe squamous papilloma in all mice, while the *Kras*^{comQ61R} allele induced a mixture of moderate and severe grade squamous papillomas (*Figure 4B,E, Figure 4—figure supplement 5, and Supplementary file 4*). As such, in these two organs, the G12D mutant was associated with more severe phenotypes than the Q61R mutant, with changing codon usage to increase protein expression shifting this difference to a more advanced stage. These data collectively support a dominant role for qualitative differences in the tumorigenic sensitivity of the upper gastrointestinal epithelium, especially at lower activity levels.

Tissues sensitive to both oncogenic activity and mutation type

The lung appeared to be a tissue whereby the effect of qualitative and quantitative signaling differences were both evident. In terms of qualitative signaling, pathological analysis performed as above revealed that the G12D mutation consistently induced more AAH and adenomas than the Q61R mutation in both the *Kras*^{com} and *Kras*^{nat} contexts (*Figure 4B,F, Figure 4—figure supplement 5,6A, and Supplementary file 4*). As was the case with forestomach and oral lesions, converting rare codons to common amplified the severity of lesions detected, which was particularly evident in the confluence of large peripheral AAH lesions induced preferentially by the G12D mutant allele (*Figure 4B,F, Figure 4—figure supplement 5, and Figure 4—figure supplement 6B*). In terms of quantitative signaling, no BH lesions were induced by either of the *Kras*^{nat} alleles and instead were only prevalent in mice in which the *Kras*^{com} alleles were activated. Further, the number of animals with BH lesions was higher upon activating the *Kras*^{comQ61R} compared to the *Kras*^{comG12D} allele (*Figure 4—figure supplement 5,6C*), akin to hematolymphopoietic neoplasia. Assuming that AAH and BH represent different types of tumors (and not different stages of the same tumor type), these data collectively support the lung being sensitive to both qualitative and quantitative oncogenic RAS signaling.

Tissues resistant to oncogenic RAS driven tumorigenesis

Despite widespread tumorigenesis, we note that no overt proliferative lesions were detected at necropsy or by histopathologic analysis in the pancreas, kidney, or liver (*Figure 4—figure supplement 5* and *Supplementary file 3*). A gross survey of other organs such as the colon, intestine, heart, skin, and mammary glands also did not reveal any macroscopically detectable tumors (not shown). In agreement, many of these same tissues were reported to be refractory to tumorigenesis upon activation of an *LSL-Kras*^{G12D} allele in the adult by CreER driven by a *Rosa26* (*Parikh et al., 2012*), *CK19* (*Ray et al., 2011*) or *Ubc9* (*Matkar et al., 2011*) promoter. Thus, many organs appear to be intrinsically resistant to the tumorigenic effects of oncogenic Kras, regardless of the mutation type or expression levels tested, at least within the timeframe of this study and in the tested *Rosa26-CreERT2* background.

Discussion

Here we describe how separating oncogenic mutations from expression level in the endogenous *Kras* gene *in vivo* revealed quantitative and qualitative signaling contributions to RAS mutation patterns. We acknowledge three caveats to this approach. First, the four oncogenic *LSL-Kras* alleles were generated by fusing coding exons 1 to 3, an artificial gene architecture, and hence can only be compared to themselves and not to other types of *Kras* alleles. Second, these alleles were induced by an injection of tamoxifen to activate CreER expressed from the *Rosa26* locus, which is admittedly an unnatural situation whereby oncogenic Kras is expressed all at once in a multitude of cells and tissues, potentially perturbing homeostasis in the whole animal. Nevertheless, as the identical design was applied to all four alleles, comparisons can be made within this allelic set, and we find that targeted activation in the lung phenocopied activation by CreER expressed from the *Rosa26* locus. Third, Kras activity of this allelic set was defined in the lung. We therefore acknowledge that cell-type differences in feedback regulatory pathways (*Lake et al., 2016; Liu et al., 2018*), or codon-dependent expression (*Peterson et al., 2020*) could result in different levels of Kras expression, activation, or signaling compared to that observed in the lung.

With these above limitations in mind, transcriptome analysis shortly after activating each allele revealed a distinct cellular response. This allelic set began with the *Kras*^{natG12D} allele, which induced EMT and differentiation hallmarks. EMT is known to promote stem cell-like fates (*Floor et al., 2011; Mani et al., 2008*) and activation of oncogenic Kras in the murine lung generates tumors with many tissue lineages (*Tata et al., 2018*). Taken together, we suggest that the transcriptional signature induced by the *Kras*^{natG12D} oncoprotein reflects either reprogramming towards or an expansion of cells with multipotent characteristics. In support, single cell transcriptome profiling of lung tumors induced by targeted delivery of AAV-Cre in an *LSL-Kras*^{G12D};*Trp53*^{fl/fl} background identified a distinct population with a mixed cellular identity (*Marjanovic et al., 2020*). At the other end of the spectrum, the *Kras*^{comQ61R} allele induced transcriptional hallmarks consistent with overt oncogenic signaling. The transition from one transcriptional profile to a completely different profile appeared to be a product of both quantitative and qualitative signaling. As the level of Kras biochemical activity increased, so did the transcriptional signatures of RAS signaling, which at its crescendo, resulted in transcriptional signatures indicative of hyperproliferative and all the hallmarks of oncogenic stress. On the other hand, we also find evidence that G12D-mutant of Kras has a distinct signature compared to that of the Q61R mutant.

Globally activating these four alleles revealed four tumorigenic patterns. First, hematolymphopoietic neoplasias were largely driven by quantitative signaling, being induced at the lowest level of oncogenic activity and increasing in aggressiveness with increased Kras activity. To independently validate this result, we tested and found that globally activating a 'super-rare' version of *LSL-Kras*^{rareG12D} allele encoded by the most rare codons possible induced hematolymphopoietic neoplasias, although in a much-protracted timeframe (*Figure 4—figure supplement 7* and *Supplementary file 3*), further supporting hematolymphopoietic tissues being hyper-sensitive to oncogenic Ras signaling. Second, proliferative lesions of forestomach and oral squamous epithelium were preferentially induced by G12D-mutant *Kras* alleles, pointing towards qualitative differences driving these tumors with higher expression shifting the effects to more aggressive grades. We acknowledge, however, that other interpretations beyond qualitative signaling differences between G12D and Q61R mutants are possible. It is worth mentioning that

the survival is similar in mice in which *Kras*^{natG12D} and *Kras*^{natQ61R} alleles were globally activated, ruling out early moribundity masking the development of squamous lesion. Nevertheless, increasing oncoprotein expression does render the Q61R mutant tumorigenic in this tissue, so it is possible that qualitative signaling differences can be overcome by robust oncogenic activity. Third, the lung was sensitive to both forms of signaling, with qualitative (G12D-specific) signaling favoring AAH and adenomas while quantitative (Ras activity) favoring BH lesions, perhaps reflecting a different cell-of-origin for these different lesions (*Sutherland et al., 2014; Xu et al., 2014*). Fourth, with the two caveats that one, the short lifespan of some of the tested mice may prevent longer latency tumors from developing, and two, even though *Rosa26*-restricted Cre expression activated the four *Kras* alleles in tissues that did not form tumors, Cre may not be expressed in the tumor cell-of-origin within these tissues, we show that many organs appear to be intrinsically resistant to the tumorigenic effects of oncogenic *Kras*, at least within the timeframe of this study and in the *Rosa26-CreERT2* background. This suggests that the hematopoietic system, lungs, forestomach, and oral mucosa are unique in being sensitive to oncogenic *Kras* tumorigenesis, again however, within the confines of the experimental design. Interestingly, these tissue sensitivities share some similarity to that of humans, namely both species have RAS-associated cancers in the lung, mouth, and hematopoietic system but not in the mammary gland, skin, central nervous system and so forth, but there is also some discordance (*Prior et al., 2020*).

Our finding that oncogenic RAS mutations are not identical in terms of activity (GTP-loading), oncogenic potential (induction of lung tumorigenesis), and cellular response (transcriptome analysis), suggests the intriguing possibility that different initiating RAS mutations may have different therapeutic sensitivities. Indeed, censoring the GSEA hallmarks for pharmacological targets already drugged in the clinic identified FLT4/PDGFRB/KIT, EGFR, and ABL1/SRC as specific to *LSL-Kras*^{natG12D} background, while AKT1/2, PI3KCA/CD/CG, SYK, RAF1, JAK2, TGFBR1, and CDK1/2/9 were specific to the *LSL-Kras*^{comQ61R} background (*Figure 5*).

In summary, we interpret these results to imply that there are tissue sensitivities to quantitative and/or qualitative RAS signaling, with quantitative signaling favoring hematolymphopoietic neoplasias, qualitative signaling favoring squamous tumors, while

carcinomas tended to be a combination of both types of signaling. The unique signaling dependencies of these tissues may, in turn, be capitalized upon to identify new therapeutic opportunities to target early tumorigenesis, when the tumors are particularly vulnerable, perhaps either as an early intervention or as a preventative measure in high-risk populations.

Materials and methods

Generation of *LSL-Kras^{natG12D}*, *LSL-Kras^{natQ61R}*, *LSL-Kras^{comG12D}*, and *LSL-Kras^{comQ61R}* alleles

A bacteria artificial chromosome was engineered with 7.5 kbp of 5' flanking sequence *Kras* intron 1 DNA, a Lox-STOP-Lox cassette (*LSL*) (*Feil et al., 1996*), exons 1 to 3 fused together and encoded by either native (*nat*) codons or with 95 rare codons converted to the most commonly used codons in the mouse genome (*com*) and either a G12D or Q61 oncogenic mutation, followed by the N-terminal 564-bp of intron 4, an FRT-Neomycin-FRT cassette, and a further 1.5 kbp of 3' flanking sequence (*Figure 1A* and *Figure 1—figure supplement 1*). The targeting cDNAs were each cloned into the targeting PL253 vector (*Liu et al., 2003*) and electroporated into 129S6/C57BL/6N (G4) ES cells. After selection, clones were screened using PCR and positive clones were selected for each engineered *Kras* allele, expanded and frozen. At least two targeted clones per allele that are confirmed with a Southern hybridization were then microinjected into blastocysts to produce chimeras using standard procedures (*Behringer et al., 2014*). *LSL-Kras^{natG12D}(+neo)*, *LSL-Kras^{natQ61R}(+neo)*, *LSL-Kras^{comG12D}(+neo)*, and *LSL-Kras^{comQ61R}(+neo)* chimeras were crossed back to 129S6 mice. A genotyping PCR specific to the engineered *Kras* alleles with *neo* cassette was used to screen for germline transmission in clones. Each 129S6-*LSL-Kras^{nat}(+neo)* and 129S6-*LSL-Kras^{com}(+neo)* cohort was crossed with *ACTB:FLPe/+* (Jackson Laboratory, strain 003800) mice to remove the selection marker via FLP-mediated excision of the *neo* cassette (*Dymecki, 1996*). Removal of the *neo* cassette was confirmed with genotyping PCR. Resultant strains were backcrossed with 129S6 mice for five generations, generating the *LSL-Kras^{natG12D}/+*, *LSL-Kras^{natQ61R}/+*, *LSL-Kras^{comG12D}/+*, and *LSL-Kras^{comQ61R}/+* strains used in this study. All mouse care and experiments were performed in accordance with a protocol approved by the Institutional Animal Care and Use Committee (IACUC) of Duke University (Protocol no. A195-19-09).

Genotyping

For genotyping, genomic DNA was isolated from 1-2 mm piece of toes by boiling for 30 min in 100 µl Toe Lysis Buffer (25 mM NaOH and 0.2 mM EDTA), followed by neutralization with 1.5 volume of Neutralization Buffer (40 mM Tris-HCl pH 6.0). 1.2 µl of the isolated genomic DNA was

subjected to PCR. Genotyping cell lines and mouse tissue was performed as above with 20 ng of genomic DNA isolated with a Qiagen DNeasy Blood and Tissue DNA Extraction Kit (Qiagen, #69504). All genotyping PCR reactions were performed using 0.4 U (0.08 µl) Platinum Taq polymerase (Invitrogen, #10342046) in 12.5 µl reaction volume with final concentration of 1.5 - 2 mM MgCl₂, 0.2 mM of each dNTP, and 0.5 µM of each primer. Full-length gels and replicates are provided (*Figure 1—source data 1* and *Figure 4—source data 1*). Mice were genotyped using the following primers:

LSL-Kras(+neo) alleles:

Kras.in3.F: 5'-TTGGTGACATCACTAGGCTTCA-3'

Kras.in3.R: 5'-TGGAAAGAGTAAAGTGTGGTGGT-3'

Kras.neo.F: 5'-GTGGGCTCTATGGCTTCTGA-3'

Products: 590 bp (Targeted Allele[+neo]) or 240 bp (WT allele)

LSL-Kras^{com} alleles:

KrasCOM5.F: 5'-CTTCCATTTGTCACGTCCTGC-3'

KrasCOM5.R: 5'-TCTTCGGTGGAAACAACGGT-3'

Product: 448 bp (LSL-Krascom)

LSL-Kras^{nat} alleles:

F-LSL: 5'-TAGTCTGTGGGACCCCTTTG-3'

R-LSL: 5'-GCCTGAAGAACGAGATCAGC-3'

Product: 448 bp (LSL-Krasnat)

Recombination PCR:

KRASOP.A2: 5'-CTAGCCACCATGGCTTGAGT-3'

KRASOP.B: 5'-GTAATCACAACAAAGAGAATGCAG-3'

LSL-F: 5'-GGGGAACCTTTCAGGCTTA-3'

Products: 616 bp (LoxP Recombined), 488 bp (WT allele), or 389 bp (*LSL* allele)

CC10-CreER alleles:

F-CC10 WT: 5'-ACTCACTATTGGGGGTGTGG-3'

R-CC10 WT: 5'-GGAGGACTTGTGGATCTTG-3'

F-Cre: 5'-TCGATGCAACGAGTGATGAG-3'

R-Cre: 5'-TTCGGCTATAGGTAACAGGG-3'

Products: 450 bp (*CC10-CreER* allele) or 350 bp (WT allele)

Rosa26-fGFP alleles:

F-Rosa-01: 5'-CACTTGCTCTTCCAAAGTCG-3'

R-Rosa-02B: 5'-TAGTCTAACTCGCGACACTG-3'

F-CAG-02B: 5'-GTTATGTAACGCGGAACTCC-3'

Products: 500 bp (WT allele allele) or 350 bp (*Rosa26-fGFP* allele)

Rosa26-CreERT2 alleles:

R26R-univF: 5'-AAAGTCGCTCTGAGTTGTTAT-3'

R26R-wtR: 5'-GGAGCGGGAGAAATGGATATG-3'

CreER-R1: 5'-CCTGATCCTGGCAATTTTCG-3'

Products: 800 bp (*Rosa26-CreERT2* allele) or 600 bp (WT allele)

LSL-Kras (LSL element):

KRASOP.A2: 5'-CTAGCCACCATGGCTTGAGT-3'

KRASOP.B: 5'-GTAATCACAACAAAGAGAATGCAG-3'

Product: 389 bp (*LSL-Kras* alleles)

CreER validation:

F-Cre: 5'-GGAGGACTTGTGGATCTTG-3'

CREER-R1: 5'-CCTGATCCTGGCAATTTTCG-3'

Product: 500 bp (CreER)

LSL-Kras^{rare} alleles:

KrasRAR.F: 5'-TATGCGTACGGGTGAAGGTT-3'

KrasRAR.R: 5'-GCAGAGCACAGACTCACGTC-3'

Product: 275 bp (*LSL-Kras^{rare}*)

Plasmids

N-terminal FLAG-tagged *Kras* cDNAs *FLAG-Kras^{natG12D}*, *FLAG-Kras^{natQ61R}*, *FLAG-Kras^{comG12D}*, and *FLAG-Kras^{comQ61R}* were designed using the same sequence of exons 1-3 as described in the engineered alleles with the addition of the native *Kras4B* to the C-terminus and cloned into pcDNA3.1+ (Thermo-Fisher). The plasmid sequences were verified by sequencing.

Mouse embryonic fibroblasts (MEFs)

LSL-Kras^{natG12D}/+, *LSL-Kras^{natQ61R}/+*, *LSL-Kras^{comG12D}/+*, and *LSL-Kras^{comQ61R}/+* mice were crossed with 129S6 mice for timed pregnancies to isolate embryos 12.5-13.5 days postcoitum, as previously described (*Serrano et al., 1997*). Two primary MEF clones from each cohort cultured in DMEM with high glucose (Sigma-Aldrich, D5796) supplemented with 10% FBS (VWR, 97068-085) were immortalized by infection with an amphotropic retrovirus derived from plasmid pBABE-neo largeTcDNA (a gift from Robert Weinberg, Addgene plasmid #1780) (*Hahn et al., 2002*) using standard methodologies (*O'Hayer & Counter, 2006*). Paired parental MEF lines that were not infected with the pBABE-neo largeTcDNA was used as a negative control for antibiotic selection. Stable immortalized MEF cultures were selected in 400 µg/ml Geneticin (Thermo-Fisher, 10131035) and again infected with an amphotrophic retrovirus derived from plasmid MSCV-Cre-Hygro (a gift from Kai Ge, Addgene plasmid #34565) to activate the mutant *Kras* allele (*Wang et al., 2010*). The stable Cre-expressing MEF cultures were selected in 500 µg/ml hygromycin (Thermo-Fisher, 10687010). Paired parental MEF lines that were not infected with MSCV-Cre-Hygro were used as negative control for hygromycin selection and for recombination PCR. Genomic DNA was isolated from the resultant cells using Qiagen DNeasy Blood and Tissue DNA Extraction Kit (Qiagen, #69504) and subjected to PCR to detect recombination of the *LSL-Kras* alleles as described above. Full-length gel image is provided (*Figure 1—source data 1*).

Ectopic expression, immunoblots, and Ras activity assay

To validate the expression levels of FLAG-tagged *Kras* constructs 2×10^6 HEK-HT cells (*Counter et al., 1992*) were seeded in 10 cm tissue culture plates in DMEM with high glucose (Sigma-Aldrich, D5796) supplemented with 10% FBS (VWR, 97068-085), and were transiently transfected the next day with the pcDNA3.1+ (empty vector) or the same plasmid encoding *FLAG-Kras^{natG12D}*, *FLAG-Kras^{natQ61R}*, *FLAG-Kras^{comG12D}*, or *FLAG-Kras^{comQ61R}* using FuGene 6 reagent (Promega, E2691) according to the manufacturer's protocol. 48 h later, transfected cells were washed with cold PBS and pelleted. Cell pellets were lysed in 5 volumes of 1x lysis buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, and 5 mM EDTA) containing Protease Inhibitor Cocktail (Roche, 11836170001). The DC Protein Assay (Bio-Rad, 5000112) was

used to measure protein concentration and 30 µg protein of each sample was resolved by SDS-PAGE, transferred to a PVDF membrane (Bio-Rad, 1704273), blocked in 5% milk, and immunoblotted by the following antibodies in 5% BSA (Sigma, A7906-500G): FLAG (Sigma, F1804; diluted 1:1000), KRAS (Santa Cruz, SC-30; diluted 1:500), and β-Tubulin (Sigma, T5201; diluted 1:10000). Primary antibody incubation was performed at room temperature for 1 h followed by the secondary antibody incubation at 4°C overnight. To measure biochemical activity of the FLAG-tagged *Kras* constructs, cells were seeded, transfected, washed with PBS as above, after which cell pellets were lysed and immediately assayed with the Active Ras Detection Kit (Cell Signaling, #8821) or Ras GTPase ELISA Kit (Abcam, ab134640) according to the manufacturer's protocol. Measurements were done with two technical replicates of four serial dilutions and data shown are representative of two independent biological replicates. Full-length images of immunoblots and replicates are provided (*Figure 1—source data 2*).

Tumorigenesis studies

LSL-Kras^{natG12D}/+, *LSL-Kras^{natQ61R}/+*, *LSL-Kras^{comG12D}/+*, and *LSL-Kras^{comQ61R}/+* mice were crossed with either *CC10-CreER/+;R26-CAG-fGFP/+* (a gift from Mark Onaitis) (*Xu et al., 2012*) or *Rosa26-CreERT2/+* (Jackson Laboratory, strain 008463) mice and animals with the desired alleles were selected by genotyping. At 6 to 8 weeks of age selected littermates with random distribution of males and females received four intraperitoneal injections of tamoxifen (Sigma-Aldrich, T5648-5G, CAS# 10540-29-1) dissolved in corn oil (Sigma-Aldrich, C8267) and filter sterilized for a dose of 250 µg/g body weight to induce Cre-mediated recombination of the *LSL* cassette, activating the engineered oncogenic mutant *Kras* alleles. Mice from the first cross were humanely euthanized 1, 2, 3, 4, 5, or 6 months later while mice from the second cross were humanely euthanized upon moribundity. To prevent age-related outcomes, four mice that didn't reach moribundity by 300 days were humanely euthanized at one year of age, which we reasoned to suffice to show tumorigenesis as it is about 8 times longer than the previously identified average moribundity of 5-6 weeks with the tamoxifen treated *Rosa26-CreERT2/+; LSL-KrasG12D/+* mice (*Parikh et al., 2012*). Selected tissues were removed at necropsy and fixed in 10% Formalin (VWR, 89370-094) for 24 to 48 h, then post-fixed in 70% ethanol (VWR, 89125-166) until analysis.

Tissue analysis

Animals were humanely euthanized with inhaled carbon dioxide and subjected to a complete necropsy. Selected organs were sampled for microscopic examination including lung, liver, kidney, spleen, thymus, stomach, pancreas, and macroscopic lesions. All tissues were fixed for 48 h in 10% neutral buffered formalin (VWR, 89370-094) and then post-fixed in 70% ethanol (VWR, 89125-166), processed routinely, embedded in paraffin with the flat sides down, sectioned at a depth of 5 μ m, and stained by the H&E method. Routine processing of the lungs from *CC10-CreER/+;R26-CAG-fGFP/+* mice was performed by the Duke Research Immunohistology Lab, while all tissues from *Rosa26-CreERT2/+* mice were processed by IDEXX Laboratories. Tissues and H&E slides were evaluated by a board certified veterinary anatomic pathologist with experience in murine pathology.

Tissue recombination analysis

At 6 to 8 weeks of age, two female mice from each of the genotypes *LSL-Kras^{natG12D}/+*, *LSL-Kras^{natQ61R}/+*, *LSL-Kras^{comG12D}/+*, and *LSL-Kras^{comQ61R}/+* in a *Rosa26-CreERT2/+* background were injected with tamoxifen as above, and seven days later humanely euthanized. One age-matched female *Rosa26-CreERT2/+; LSL-Kras^{comG12D}* mouse was also humanely euthanized as a no-tamoxifen control. At necropsy the colon, duodenum, ileum, cecum, jejunum, pancreas, spleen, glandular stomach, forestomach, kidney, liver, lung, heart, and ovaries were removed, genomic DNA extracted, and the status of recombination of each of the four oncogenic mutant *LSL-Kras* alleles determined by recombination PCR in duplicate, as described above. The intensities of bands corresponding to the unaltered wild-type *Kras* allele (WT) as well as the unrecombined and recombined oncogenic mutant *LSL-Kras* alleles was quantified with ImageJ software and the recombination rates for each tissue type from each mouse calculated by dividing the densitometry of recombined allele to that of the WT *Kras* allele. Full-length gels and replicates are provided (*Figure 4—source data 1*).

Codon usage plots

The codon usage index (*Sharp & Li, 1987*) was calculated using the relative codon frequency derived from codon usage in the mouse exome (*Nakamura et al., 2000*) with a sliding windows

of 25 codons across the open reading frame (ORF) of each transcript. A theoretical *Kras* ORF encoded by the rarest codons at each position (grey dotted line) was plotted for reference (*Figure 1—figure supplement 1*).

Statistical analysis

Statistical analyses were performed using GraphPad Prism software, version 8 (GraphPad Software). One-way ANOVA with Bonferroni's multiple comparisons test with a single pooled variance and a 95% CI were used for experiments with more than 2 groups. Reported *P* values are adjusted to account for multiple comparisons. A *P* value of less than 0.05 was considered statistically significant.

RNAseq

Three mice with random distribution of males and females were selected from cohorts of *LSL-Kras^{natG12D}/+*, *LSL-Kras^{natQ61R}/+*, *LSL-Kras^{comG12D}/+*, and *LSL-Kras^{comQ61R}/+* mice in a *Rosa26-CreERT2/+* background, injected with tamoxifen at 6 to 8 weeks of age as described above, and humanely euthanized seven days later to harvest lungs. Three mice with random distribution of males and females in *Rosa26-CreERT2/+* background without the *LSL-Kras* alleles were used as negative control. For the second RNAseq experiment, three mice with random distribution of males and females were selected from *LSL-Kras^{natG12D}/+*, and *LSL-Kras^{comQ61R}/+* mice in a *Rosa26-CreERT2/+* background, injected with tamoxifen at 6 to 8 weeks of age as described above, and humanely euthanized seven days later to harvest lungs. All tissue lysis and RNA extraction steps were performed in a chemical hood and all instruments and tools were sprayed with RNaseZAP™ (Sigma, R2020) to prevent RNA degradation. Isolated lungs were immediately stored in RNA stabilizing solution RNAlater (Sigma, R0901) at 4°C overnight and then transferred to -80°C for long-term storage. Lung tissues were thawed, weighed, and pulverized with mortar and pestle in the presence of liquid nitrogen. RNA extraction was performed immediately thereafter using RNeasy Kit (Qiagen, 74104) according to manufacturer's instructions. Briefly, tissue lysates were prepared according to the kit instructions and tissue clumps were removed using QIAshredder columns (Qiagen, 79654). Cleared lysates were applied to RNeasy silica columns and on-column DNase digestion was performed using RNase-Free DNase Set (QIAGEN, 79254) to remove DNA in silica membrane. Following wash steps, RNA was eluted in RNase-free water that

was treated with RNAsecure™ RNase Inactivation Reagent (Thermo-Fisher, AM7005) according to manufacturer's instructions. Extracted total RNA quality and concentration was assessed on Fragment Analyzer (Agilent Technologies) and Qubit 2.0 (ThermoFisher Scientific), respectively. Samples with RIN less than 7 were not sequenced. RNA-seq libraries were prepared using the commercially available KAPA Stranded mRNA-Seq Kit (Roche). In brief, mRNA transcripts were captured from 500 ng of total RNA using magnetic oligo-dT bead. The mRNA was then fragmented using heat and magnesium, and reverse transcribed using random priming. During second strand synthesis, the cDNA:RNA hybrid was converted into to double-stranded cDNA (dscDNA) and dUTP was incorporated into the second cDNA strand, effectively marking this strand. Illumina sequencing adapters were ligated to the dscDNA fragments and amplified to produce the final RNA-seq library. The strand marked with dUTP was not amplified, allowing strand-specificity sequencing. Libraries were indexed using a dual indexing approach allowing for all the libraries to be pooled and sequenced on the same sequencing run. Before pooling and sequencing, fragment length distribution for each library was first assessed on a Fragment Analyzer (Agilent Technologies). Libraries were also quantified using Qubit. Molarity of each library was calculated based on qubit concentration and average library size. All libraries were then pooled in equimolar ratio and sequenced. Sequencing was done on an Illumina NovaSeq 6000 sequencer. The pooled libraries were sequenced on one lane of an S-Prime flow cell at 50bp paired-end. Once generated, sequence data was demultiplexed and Fastq files generated using bcl2fastq v2.20.0.422 file converter from Illumina. The RNAseq data has been deposited in NCBI's Gene Expression Omnibus (*Edgar et al., 2002*). Initial and secondary RNAseq data are available through GEO Series accession numbers *GSE181628* and *GSE181627*, respectively, and are accessible under the project *GSE181629* (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE181629>).

Transcriptome analysis

RNA-seq data was processed using the TrimGalore toolkit (http://www.bioinformatics.babraham.ac.uk/projects/trim_galore) which employs Cutadapt (*Martin, 2011*) to trim low-quality bases and Illumina sequencing adapters from the 3' end of the reads. Only reads that were 20 nucleotides or longer after trimming were kept for further analysis. Reads were mapped

to the GRCm38v73 version of the mouse genome and transcriptome (Kersey et al., 2012) using the STAR RNA-seq alignment tool (Dobin et al., 2013). Reads were kept for subsequent analysis if they mapped to a single genomic location. Gene counts were compiled using the HTSeq tool (<http://www-huber.embl.de/users/anders/HTSeq/>). Only genes that had at least 10 reads in any given library were used in subsequent analysis. Normalization and differential expression was carried out using the DESeq2 (Love et al., 2014) Bioconductor (Huber et al., 2015) package with the R statistical programming environment (<http://www.R-project.org>). The false discovery rate was calculated to control for multiple hypothesis testing (Figure 3—figure supplement 2-5). Gene set enrichment analysis (Mootha et al., 2003) was performed to identify hallmarks and pathways associated with altered gene expression for each of the comparisons performed (Figure 3—figure supplement 6 and Figure 3—source data 1). To identify low Kras- and high Kras-specific druggable kinases, the transcriptome enriched in *Kras^{natG12D}/+* and *Kras^{comQ61R}/+*-specific GSEA hallmarks were cross referenced with kinases whose clinical inhibitors were previously surveyed (Klaeger et al., 2017). The kinases enriched in *Kras^{natG12D}/+* and in *Kras^{comQ61R}/+* are shown in blue and red, respectively with adjusted *p*-value less than 5% were highlighted (Figure 5).

Quantitative Real-Time PCR

Two mice with random distribution of males and females were selected from cohorts of *LSL-Kras^{natG12D}/+*, *LSL-Kras^{natQ61R}/+*, *LSL-Kras^{comG12D}/+*, and *LSL-Kras^{comQ61R}/+* mice in a *Rosa26-CreERT2/+* background, injected with tamoxifen at 6 to 8 weeks of age as described above, and humanely euthanized seven days later to harvest lungs. Three mice with random distribution of males and females in *Rosa26-CreERT2/+* background without the *LSL-Kras* alleles were used as negative control. RNA extraction was performed as mentioned above, followed by first strand cDNA synthesis from 2 µg RNA, and real-time PCR using GoTaq 2-Step RT-qPCR kit (Promega, A6110). All measurements were normalized against Actin as the internal control using the $2^{-\Delta\Delta Ct}$ method (Figure 1—source data 3 and Figure 3—source data 2). For qRT-PCR analysis for the second biological replicate, one of two negative controls did not have numerical data for two transcripts and hence was not used for plotting. Data shown are representative of two independent biological replicates and the primer sequences are:

GLI1-F: 5'-CCCATAGGGTCTCGGGGTCTCAAAC-3', GLI1-R: 5'-GGAGGACCTGCGGCTGACTGTGTAA-3'
 CDH1-F: 5'-GTCTCCTCATGGCTTTGC-3', ECAD-R: 5'-CTTTAGATGCCGCTTCAC-3'
 TWIST1-F: 5'-AGCGGGTCATGGCTAACG-3', TWIST1-R: 5'-GGACCTGGTACAGGAAGTCGA-3'
 ZEB2-F: 5'-GAGCTTGACCACCGACTC-3', ZEB2-R: 5'-TTGCAGGACTGCCTTGAT-3'
 SOX5-F: 5'-ATTGTGCAGTCCCACAGGTTG-3', SOX5-R: 5'-CTGCCTTTAGTGGGCCAGTG-3'
 IL6-F: 5'-CCGGAGAGGAGACTTCACAG-3', IL6-R: 5'-CAGAATTGCCATTGCACAAC-3'
 IL10-F: 5'-GGTTGCCAAGCCTTATCGGA-3', IL10-R: 5'-ACCTGCTCCACTGCCTTGCT-3'
 TNFA-F: 5'-CCCCAAAGGGATGAGAAGTT-3', TNFA-R: 5'-GTGGGTGAGGAGCACGTAGT-3'
 CCL2-F: 5'-AGGTCCCTGTCATGCTTCTG-3', CCL2-R: 5'-TCTGGACCCATTCTTCTTG-3'
 LIF-F: 5'-AATGCCACCTGTGCCATACG-3', LIF-R: 5'-CAACTTGGTCTTCTCTGTCCCG-3'
 TNFSF9-F: 5'-GCAAGCAAAGCCTCAGGTAG-3', TNFSF9-R: 5'-TCCAGGAACGGTCCACTAAC-3'
 IFNG-F: 5'-CGGGAGGTGCTGCTGATGG-3', IFNG-R: 5'-AGGGACAGCCTGTTACTACC-3'

Generation of *LSL-Kras^{rareG12D}/+* mice and tumorigenesis study

An additional inducible *Kras* allele with the most rare codons was generated in the same manner as mentioned above for the four *LSL-Kras* alleles from BAC design to chimera production (**Figure 1—figure supplement 1**). *LSL-Kras^{rareG12D}(+neo)* chimeras were crossed back to 129S6 mice followed by *ACTB:FLPe/+* (Jackson Laboratory, strain 003800) mice to remove the *neo* selection marker via FLP-mediated excision (**Dymecki, 1996**). Both germline transmission and the removal of the *neo* cassette were confirmed with genotyping PCR as mentioned above. Resultant strain was backcrossed with 129S6 mice for five generations, generating the *LSL-Kras^{rareG12D}/+* strain. *LSL-Kras^{rareG12D}/+* mice were crossed with *Rosa26-CreERT2/+* (Jackson Laboratory, strain 008463) mice and animals with the desired alleles selected by genotyping. At 6 to 8 weeks of age seven male and female adult mice received four intraperitoneal injections of tamoxifen (Sigma-Aldrich, T5648-5G, CAS# 10540-29-1) dissolved in corn oil (Sigma-Aldrich, C8267) and filter sterilized for a dose of 250 µg/g body weight to activate the engineered oncogenic mutant *Kras* allele. Tamoxifen injected mice were humanely euthanized at the fixed timepoint of 22 months later with inhaled carbon dioxide. Lung, liver, kidney, spleen, thymus, stomach, pancreas, and femurs were removed at necropsy and fixed in 10% Formalin (VWR, 89370-094) for 24 to 48 h, then post-fixed in 70% ethanol (VWR, 89125-166), processed routinely, embedded in paraffin with the flat

sides down, sectioned at 5 µm, and stained with H&E. Routine processing of the tissues was performed by IDEXX Laboratories. Tissues and H&E slides were evaluated by a board certified veterinary anatomic pathologist with experience in murine pathology.

Online Supplemental Material

Figure 1—figure supplement 1 shows the codon usage index versus codon number of *Kras* with native, common, and rare codons. **Figure 1—figure supplement 2** shows the sequence alignment of the coding exons of the four novel *LSL-Kras* alleles used in this study. **Figure 1—figure supplement 3** shows protein expression and activity levels of the encoded *Kras* proteins. **Figure 1—figure supplement 4** shows the qRT-PCR analysis of the RAS target genes. **Figure 2—figure supplement 1** shows the lung-specific activation of the four *LSL-Kras* alleles. **Figure 3—figure supplement 1** shows transcriptomic analysis of the lung immediately after activation of the four *LSL-Kras* alleles. **Figure 3—figure supplement 2-5** shows the volcano plot of the $\log_2$ fold-change versus p value of differentially expressed genes from RNA-seq analysis of the lungs of *Rosa26-CreERT2/+;Kras+/+* mice versus **figure supplement 2** *Rosa26-CreERT2/+;LSL-Kras^{natG12D}/+*, **figure supplement 3** *Rosa26-CreERT2/+;LSL-Kras^{natQ61R}/+*, **figure supplement 4** *Rosa26-CreERT2/+;LSL-Kras^{comG12D}/+*, and **figure supplement 5** *Rosa26-CreERT2/+;LSL-Kras^{comQ61R}/+* mice. **Figure 3—figure supplement 6** shows GSEA hallmarks regulated by the four *LSL-Kras* alleles. **Figure 3—figure supplement 7** shows validation of the transcriptome via qRT-PCR analysis of selected genes. **Figure 4—figure supplement 1** shows the experimental design to determine tissue sensitivities of the four *LSL-Kras* alleles. **Figure 4—figure supplement 2** shows the recombination analysis of 14 organs after tamoxifen injection. **Figure 4—figure supplement 3** shows the number of different tumor types detected in each allele at the moribundity endpoint. **Figure 4—figure supplement 4** shows pairwise comparisons of the survival curves based on Log-Rank (Mantel Cox) test. **Figure 4—figure supplement 5** shows the examples of H&E-stained tissues from multiple organs from each of the four *LSL-Kras* alleles. **Figure 4—figure supplement 6** analysis of pulmonary lesions in the *Rosa26-CreERT2/+* background. **Figure 4—figure supplement 7** shows the tumor landscape after activating the ‘super-rare’ *LSL-Kras^{rareG12D}* 20 months after tamoxifen injection.

Figure 1—source data 1 shows the full-length gel images of genotyping of MEF cultures in **Figure 1B**. **Figure 1—source data 2** shows the full-length gel images of immunoblots in **Figure 1—figure supplement 3**. **Figure 1—source data 3** shows the Ct values from the qRT-PCR analysis in **Figure 1—figure supplement 4**. **Figure 3—source data 1** shows the normalized enrichment

scores of the hallmarks identified in *Figure 3—figure supplement 6*. *Figure 3—source data 2* shows the Ct values from the qRT-PCR analysis in *Figure 1—figure supplement 4* and *Figure 3—figure supplement 7*. *Figure 4—source data 1* shows the full-length gel images of recombination analysis in *Figure 4—figure supplement 2*.

Supplementary file 1 shows the gel densitometer results of recombination analysis of 14 organs after tamoxifen injection in *Figure 4—figure supplement 2*. *Supplementary file 2* shows the statistical analysis of pairwise comparisons of the survival plots of the four *LSL-Kras* alleles in *Figure 4A*. *Supplementary file 3* shows the histopathology report for H&E stained tissues from multiple organs from each of the four *LSL-Kras* alleles. *Supplementary file 4* shows the grading of observed tumors in multiple organs from each of the four *LSL-Kras* alleles.

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Author Contributions: O. Erdogan, N.L.K. Pershing, E. Kaltenbrun, and C.M. Counter conceived the study. O. Erdogan and C.M. Counter designed the experiments and the methodology. O. Erdogan and E. Kaltenbrun established the mouse models. O. Erdogan performed the tumor studies, cell culture experiments, RAS activity assays, tissue isolation, RNA extraction, and RNASeq. N.J. Newman performed recombination PCR. O. Erdogan analyzed recombination PCR data, tumor studies, and transcriptome data. J.I. Everitt analyzed the histopathology. C.M.

Counter supervised the study. O. Erdogan, N.L.K. Pershing, J.I. Everitt, and C.M. Counter wrote the manuscript. All authors read, edited, and agreed on the final version of the manuscript.

Competing interests

CMC is the co-founder of Merlon Inc, which played no role in the design, experiments, or data analysis of this study.

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Figure Legends

Figure 1. **Conditional *LSL-Kras* alleles with different oncogenic mutations and codon usage.**

(A) Schematic of generating and activating *LSL-Kras* alleles with coding exons 1 to 3 encoded by native (*nat*) versus common (*com*) codons with either a G12D or Q61R mutation.

(B) PCR genotyping of two independently derived MEF cultures (two biological replicates) with the indicated *LSL-Kras* alleles in the absence and presence of Cre recombinase (CRE) to detect the unaltered wild-type *Kras* allele product (WT, 488 bp) as well as the unrecombined (*LSL-Kras**, 389 bp) and recombined (LoxP recombined, 616 bp) *LSL-Kras* allelic products. Gel images were cropped and color inverted for better visualization. Full-length gel images are provided in **Figure 1—source data 1**.

(C) Levels of active KRAS determined by RBD pull-down (RBD-PD) followed by ELISA analysis using lysates derived from HEK-HT cells transiently expressing the indicated FLAG-tagged *Kras* proteins. Tubulin and empty vector serve as loading and negative controls, respectively. One of two biological replicates, see **Figure 1—figure supplement 4C** for the second biological replicate.

Figure 2. **Biological effect of each oncogenic *LSL-Kras* allele.**

(A) Examples of H&E-stained lung sections, and **(B)** the mean $\pm$ SD % tumor burden from microscopic analysis of two lung sections from five mice with the indicated *LSL-Kras* alleles in a *CC10-CreER/+* background at each of the indicated times post-tamoxifen injection.

Figure 3. **Transcriptomes of each oncogenic *LSL-Kras* allele.**

(A) Volcano plot of the $\log_2$ fold-change versus *p* value of the genes showing differential expression in each allele compared to the wild-type *Kras* allele in the lung. Full-sized plots are provided in **Figure 3—figure supplement 2-5**.

(B) STRING analysis of the top ten genes in the differentially enriched GSEA hallmarks identified in RNA-seq analysis of the lungs of *Rosa26-CreERT2/+;LSL-Kras^{natG12D}* versus *Rosa26-CreERT2/+;LSL-Kras^{comQ61R}* mice seven days after tamoxifen injection.

(C,D) Normalized enrichment score and patterns of the indicated GSEA hallmarks regulated by the quantitative **(C)** and qualitative **(D)** RAS signaling. Only hallmarks with an FDR less than 5% are shown. Dot size is adjusted to RAS activity for better visualization. All GSEA Hallmarks differentially regulated by quantitative and qualitative Ras signaling are provided in **Figure 3—figure supplement 6**.

Figure 4. **Tissue atlas of sensitivities to each oncogenic *LSL-Kras* allele.**

(A) Kaplan-Meier survival curve of the indicated *LSL-Kras* alleles after activation by tamoxifen. Dotted lines: 50% survival.

(B) Number of mice with the indicated number of different tumor types. Examples of H&E-stained slides of the indicated tissues are provided in **Figure 4—figure supplement 5**.

(C-F) Percentage of the indicated grades of **(C)** hematolymphopoietic, **(D)** forestomach, **(E)** oral, and **(F)** lung lesions at moribundity endpoint in *Rosa26-CreERT2/+* mice ($n= 8$ to 10) with one of the four indicated *LSL-Kras* alleles after activation by tamoxifen.

Figure 5. **Transcriptome analysis predicts pharmacologic vulnerabilities of different oncogenic *LSL-Kras* alleles.**

Druggable kinases positively enriched in GSEA hallmarks identified in **Figure 4B** (blue *Kras^{natG12D}*, red *Kras^{comQ61R}*). * adjusted p -value less than 5%.

KEY RESOURCE TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
FLAG	Sigma	F1804
KRAS	Santa Cruz Biotechnology	sc-30
Beta-Tubulin	Sigma	T5201
Bacteria strains		
STBL3	Thermo Fisher Scientific	C737303
Chemicals, peptides, recombinant proteins		
DMEM with high glucose	Sigma-Aldrich	D5796
Fetal Bovine Serum	VWR	97068-085
FuGENE® 6 Transfection Reagent	Promega	E2691
Protease inhibitor cocktail	Roche	11836170001
DC Protein Assay	Bio-Rad	5000112
Geneticin	Thermo-Fisher	10131035
Hygromycin	Thermo-Fisher	10687010
DNeasy Blood and Tissue DNA extraction Kit	Qiagen	69504
Platinum Taq polymerase	Invitrogen	10342046
Tamoxifen	Sigma-Aldrich	T5648-5G
Corn oil	Sigma-Aldrich	C8267
RNaseZAP™	Sigma-Aldrich	R2020
RNA Later	Sigma-Aldrich	R0901
RNAeasy Kit	Qiagen	74104
QIAshredder columns	Qiagen	79654
RNase-Free DNase Set	Qiagen	79254
RNAsecure™ RNase Inactivation Reagent	Thermo-Fisher	AM7005
Critical Commercial Assays		
Active Ras Detection Kit	Cell Signaling	8821
Ras GTPase ELISA Kit	Abcam	ab134640
Cell lines		
HEK-HT	(Counter et al., 1992)	
Mouse Strains		
LSL-Kras ^{natG12D} /+	This study	
LSL-Kras ^{natQ61R} /+	This study	
LSL-Kras ^{comG12D} /+	This study	
LSL-Kras ^{comQ61R} /+	This study	
ACTB:FLPe/+	Jackson Laboratory	003800
CC10-CreER/+;R26-CAG-fGFP/+	(Xu et al., 2012)	
Rosa26-CreERT2/+	Jackson Laboratory	008463
Recombinant DNA		
pcDNA3.1+ FLAG-Kras ^{natG12D}	This study	
pcDNA3.1+ FLAG-Kras ^{natQ61R}	This study	
pcDNA3.1+ FLAG-Kras ^{comG12D}	This study	
pcDNA3.1+ FLAG-Kras ^{comQ61R}	This study	
pBABE-neo largeTcDNA	(Hahn et al., 2002)	Addgene #1780
MSCV-Cre-Hygro	(Wang et al., 2010)	Addgene #34565
Software and algorithms		
ImageJ version 1.52k with Java 1.8.0_172	NIH (Schneider et al., 2012)	https:// imagej.nih.gov/ij/

Image Lab	Bio-Rad	https://www.bio-rad.com/en-us/product/image-lab-software
Graphpad Prism v8	Graphpad	https://www.graphpad.com/
bcl2fastq	Illumina	https://support.illumina.com/sequencing/sequencing_software/bcl2fastq-conversion-software.html
STAR RNA-Seq alignment tool	(<i>Dobin et al., 2013</i>)	https://github.com/alexdobin/STAR
DESeq2	(<i>Love et al., 2014</i>)	http://www.bioconductor.org/packages/release/bioc/html/DESeq2.html
GSEA	(<i>Mootha et al., 2003</i>)	https://www.gsea-msigdb.org/gsea/
R Studio		https://www.R-project.org

Figure 1

A

B

C

Figure 2

A

B

Figure 3

A

B Enriched in *Kras*^{natG12D/+}

Enriched in *Kras*^{comQ61R/+}

C

Quantitative Signaling

D

Qualitative Signaling

Figure 4

Figure 5

Figure 1—figure supplement 1

Figure 1—figure supplement 1. Codon usage of the four novel *LSL-Kras* alleles.

Codon usage index versus codon number of *Kras* with native (nat, blue line), common (com, red line) and rare (rare, orange line) codons. Maximum rare (all rare, grey dotted line) and common (all common, solid grey line) codon usage are shown for reference.

Figure 1—figure supplement 2

	M	T	E	Y	K	L	V	V	V	G	A	G	G	V	G	K	S	A	L	T	
mKras	ATG	ACT	GAG	TAT	AAA	CTT	GTG	GTG	GTT	GGA	GCT	GGT	GGC	GTA	GGC	AAG	AGC	GCC	TTG	ACG	60
natG12D	ATG	ACT	GAG	TAT	AAA	CTT	GTG	GTG	GTT	GGA	GCT	GAT	GGC	GTA	GGC	AAG	AGC	GCC	TTG	ACG	60
natQ61R	ATG	ACT	GAG	TAT	AAA	CTT	GTG	GTG	GTT	GGA	GCT	GGT	GGC	GTA	GGC	AAG	AGC	GCC	TTG	ACG	60
comG12D	ATG	ACC	GAG	TAC	AAG	CTG	GTG	GTG	GTG	GGC	GCC	GAC	GGC	GTA	GGC	AAG	AGC	GCC	CTG	ACC	60
comQ61R	ATG	ACC	GAG	TAC	AAG	CTG	GTG	GTG	GTG	GGC	GCC	GGT	GGC	GTA	GGC	AAG	AGC	GCC	CTG	ACC	60
	***	**	***	**	**	**	***	***	***	**	**	*	***	**	***	***	***	***	**	**	
	I	Q	L	I	Q	N	H	F	V	D	E	Y	D	P	T	I	E	D	S	Y	
mKras	ATA	CAG	CTA	ATT	CAG	AAT	CAC	TTT	GTG	GAT	GAG	TAT	GAC	CCT	ACG	ATA	GAG	GAC	TCC	TAC	120
natG12D	ATA	CAG	CTA	ATT	CAG	AAT	CAC	TTT	GTG	GAT	GAG	TAC	GAC	CCT	ACG	ATA	GAG	GAC	TCC	TAC	120
natQ61R	ATA	CAG	CTA	ATT	CAG	AAT	CAC	TTT	GTG	GAT	GAG	TAC	GAC	CCT	ACG	ATA	GAG	GAC	TCC	TAC	120
comG12D	ATC	CAG	CTG	ATC	CAG	AAC	CAC	TTT	GTG	GAC	GAG	TAC	GAC	CCT	ACC	ATC	GAG	GAC	AGC	TAC	120
comQ61R	ATC	CAG	CTG	ATC	CAG	AAC	CAC	TTC	GTG	GAC	GAG	TAC	GAC	CCT	ACC	ATC	GAG	GAC	AGC	TAC	120
	**	***	**	**	***	**	***	**	***	**	***	**	***	**	**	**	***	***	*	***	
	R	K	Q	V	V	I	D	G	E	T	C	L	L	D	I	L	D	T	A	G	
mKras	AGG	AAA	CAA	GTA	GTA	ATT	GAT	GGA	GAA	ACC	TGT	CTC	TTG	GAT	ATT	CTC	GAC	ACA	GCA	GGT	180
natG12D	AGG	AAA	CAA	GTA	GTA	ATT	GAT	GGA	GAA	ACC	TGT	CTC	TTG	GAT	ATT	CTC	GAC	ACA	GCA	GGT	180
natQ61R	AGG	AAA	CAA	GTA	GTA	ATT	GAT	GGA	GAA	ACC	TGT	CTC	TTG	GAT	ATT	CTC	GAC	ACA	GCA	GGT	180
comG12D	AGG	AAG	CAG	GTG	GTG	ATC	GAC	GGC	GAG	ACC	TGC	CTG	CTG	GAC	ATC	CTG	GAC	ACC	GCC	GGC	180
comQ61R	AGG	AAG	CAG	GTG	GTG	ATC	GAC	GGC	GAG	ACC	TGC	CTG	CTG	GAC	ATC	CTG	GAC	ACC	GCC	GGC	180
	***	**	**	**	**	**	**	**	**	***	**	**	**	**	**	**	***	**	**	**	
	Q	E	E	Y	S	A	M	R	D	Q	Y	M	R	T	G	E	G	F	L	C	
mKras	CAA	GAG	GAG	TAC	AGT	GCA	ATG	AGG	GAC	CAG	TAC	ATG	AGA	ACT	GGG	GAG	GGC	TTT	CTT	TGT	240
natG12D	CAA	GAG	GAG	TAC	AGT	GCA	ATG	AGG	GAC	CAG	TAC	ATG	AGA	ACT	GGG	GAG	GGC	TTT	CTT	TGT	240
natQ61R	CGG	GAG	GAG	TAC	AGT	GCA	ATG	AGG	GAC	CAG	TAC	ATG	AGA	ACT	GGG	GAG	GGC	TTT	CTT	TGT	240
comG12D	CAG	GAG	GAG	TAC	AGC	GCC	ATG	AGG	GAC	CAG	TAC	ATG	AGG	ACC	GGC	GAG	GGC	TTT	CTG	TGC	240
comQ61R	CGG	GAG	GAG	TAC	AGC	GCC	ATG	AGG	GAC	CAG	TAC	ATG	AGG	ACC	GGC	GAG	GGC	TTT	CTG	TGC	240
	*	***	***	***	**	**	***	***	***	***	***	***	**	**	**	***	***	**	**	**	
	V	F	A	I	N	N	T	K	S	F	E	D	I	H	H	Y	R	E	Q	I	
mKras	GTA	TTT	GCC	ATA	AAT	AAT	ACT	AAA	TCA	TTT	GAA	GAT	ATT	CAC	CAT	TAT	AGA	GAA	CAA	ATT	300
natG12D	GTA	TTT	GCC	ATA	AAT	AAT	ACT	AAA	TCA	TTT	GAA	GAT	ATT	CAC	CAT	TAT	AGA	GAA	CAA	ATT	300
natQ61R	GTA	TTT	GCC	ATA	AAT	AAT	ACT	AAA	TCA	TTT	GAA	GAT	ATT	CAC	CAT	TAT	AGA	GAA	CAA	ATT	300
comG12D	GTG	TTT	GCC	ATC	AAC	AAC	ACC	AAG	AGC	TTT	GAG	GAC	ATC	CAC	CAC	TAC	AGG	GAG	CAG	ATC	300
comQ61R	GTG	TTT	GCC	ATC	AAC	AAC	ACC	AAG	AGC	TTT	GAG	GAC	ATC	CAC	CAC	TAC	AGG	GAG	CAG	ATC	300
	**	**	***	**	**	**	**	**	**	**	**	**	**	***	**	**	**	**	**	**	
	K	R	V	K	D	S	E	D	V	P	M	V	L	V	G	N	K	C	D	L	
mKras	AAA	AGA	GTA	AAG	GAC	TCT	GAA	GAT	GTG	CCT	ATG	GTC	CTG	GTA	GGG	AAT	AAG	TGT	GAT	TTG	360
natG12D	AAA	AGA	GTA	AAG	GAC	TCT	GAA	GAT	GTG	CCT	ATG	GTC	CTG	GTA	GGG	AAT	AAG	TGT	GAT	TTG	360
natQ61R	AAA	AGA	GTA	AAG	GAC	TCT	GAA	GAT	GTG	CCT	ATG	GTC	CTG	GTA	GGG	AAT	AAG	TGT	GAT	TTG	360
comG12D	AAG	AGG	GTG	AAG	GAC	AGC	GAG	GAC	GTG	CCT	ATG	GTG	CTG	GTG	GGC	AAC	AAG	TGC	GAC	CTG	360
comQ61R	AAG	AGG	GTG	AAG	GAC	AGC	GAG	GAC	GTG	CCT	ATG	GTG	CTG	GTG	GGC	AAC	AAG	TGC	GAC	CTG	360
	**	**	**	***	***	**	**	**	***	***	***	**	***	**	**	**	***	**	**	**	
	P	S	R	T	V	D	T	K	Q	A	Q	E	L	A	R	S	Y	G	I	P	
mKras	CCT	TCT	AGA	ACA	GTA	GAC	ACG	AAA	CAG	GCT	CAG	GAG	TTA	GCA	AGG	AGT	TAC	GGG	ATT	CCG	420
natG12D	CCT	TCT	AGA	ACA	GTA	GAC	ACG	AAA	CAG	GCT	CAG	GAG	TTA	GCA	AGG	AGT	TAC	GGG	ATT	CCG	420
natQ61R	CCT	TCT	AGA	ACA	GTA	GAC	ACG	AAA	CAG	GCT	CAG	GAG	TTA	GCA	AGG	AGT	TAC	GGG	ATT	CCG	420
comG12D	CCT	AGC	AGG	ACC	GTG	GAC	ACC	AAG	CAG	GCC	CAG	GAG	CTG	GCC	AGG	AGC	TAC	GGC	ATC	CCT	420
comQ61R	CCT	AGC	AGG	ACC	GTG	GAC	ACC	AAG	CAG	GCC	CAG	GAG	CTG	GCC	AGG	AGC	TAC	GGC	ATC	CCT	420
	***	**	**	**	***	***	**	**	***	**	***	**	**	**	***	**	***	**	**	**	
	F	I	E	T	S	A	K	T	R	Q	G	V	D	D	A	F	Y	T	L	V	
mKras	TTC	ATT	GAG	ACC	TCA	GCA	AAG	ACA	AGA	CAG	GGT	GTT	GAC	GAT	GCC	TTC	TAT	ACA	TTA	GTC	480
natG12D	TTC	ATT	GAG	ACC	TCA	GCA	AAG	ACA	AGA	CAG	GGT	GTT	GAC	GAT	GCC	TTC	TAT	ACA	TTA	GTC	480
natQ61R	TTC	ATT	GAG	ACC	TCA	GCA	AAG	ACA	AGA	CAG	GGT	GTT	GAC	GAT	GCC	TTC	TAT	ACA	TTA	GTC	480
comG12D	TTC	ATC	GAG	ACC	AGC	GCC	AAG	ACC	AGG	CAG	GGT	GTT	GAC	GAT	GCC	TTC	TAT	ACA	TTA	GTC	480
comQ61R	TTC	ATC	GAG	ACC	AGC	GCC	AAG	ACC	AGG	CAG	GGT	GTT	GAC	GAT	GCC	TTC	TAT	ACA	TTA	GTC	480
	***	**	***	***	**	***	**	**	**	***	***	***	***	***	***	***	***	***	***	***	
	R	E	I	R	K	H	K	E	K	M	S	K	D	G	K	K	K	K	K	K	
mKras	CGA	GAA	ATT	CGA	AAA	CAT	AAA	GAA	AAG	ATG	AGC	AAA	GAT	GGG	AAG	AAG	AAG	AAG	AAG	AAG	540
natG12D	CGA	GAA	ATT	CGA	AAA	CAT	AAA	GAA	AAG	ATG	AGC	AAA	GAT	GGG	AAG	AAG	AAG	AAG	AAG	AAG	540
natQ61R	CGA	GAA	ATT	CGA	AAA	CAT	AAA	GAA	AAG	ATG	AGC	AAA	GAT	GGG	AAG	AAG	AAG	AAG	AAG	AAG	540
comG12D	CGA	GAA	ATT	CGA	AAA	CAT	AAA	GAA	AAG	ATG	AGC	AAA	GAT	GGG	AAG	AAG	AAG	AAG	AAG	AAG	540
comQ61R	CGA	GAA	ATT	CGA	AAA	CAT	AAA	GAA	AAG	ATG	AGC	AAA	GAT	GGG	AAG	AAG	AAG	AAG	AAG	AAG	540
	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	
	S	R	T	R	C	T	V	M	-												
mKras	TCA	AGG	ACA	AGG	TGT	ACA	GTT	ATG	TGA												
natG12D	TCA	AGG	ACA	AGG	TGT	ACA	GTT	ATG	TGA												
natQ61R	TCA	AGG	ACA	AGG	TGT	ACA	GTT	ATG	TGA												
comG12D	TCA	AGG	ACA	AGG	TGT	ACA	GTT	ATG	TGA												
comQ61R	TCA	AGG	ACA	AGG	TGT	ACA	GTT	ATG	TGA												
	***	***	***	***	***	***	***	***	***												

Figure 1—figure supplement 2. Coding sequence of exons 1 to 3 of the four novel *LSL-Kras* alleles.

Sequence alignment of the codons in the coding exons 1-3 of the indicated *LSL-Kras* alleles in comparison to mouse *Kras* transcript (*mKras*). *Top*: amino acid sequence. *Red nucleotides*: optimized codons. *Green highlight*: G12D mutation. *Blue highlight*: Q61R mutation.

Figure 1—figure supplement 3

Figure 1—figure supplement 3. Expression and activity level of the four novel encoded *Kras* proteins.

Expression levels, determined by immunoblot with an anti-FLAG antibody, and activity levels, determined by RBD pull-down (RBD-PD) followed by (A,B) immunoblots of lysates derived from HEK-HT cells transiently expressing the indicated FLAG-tagged *Kras* proteins and (C) by ELISA as a biological replicate of **Figure 1C**. EV: Empty Vector. A and B show two biological replicates. Tubulin and empty vector serve as loading and negative controls, respectively. Full-length gel images are provided at **Figure 1—source data 2**.

A

Figure 1—figure supplement 4

B **C** **Figure 1—figure supplement 4. qRT-PCR validates an increase in RAS target gene expression with increased Kras activity.**

2 mice from cohorts of *LSL-Kras^{natG12D/+}*, *LSL-Kras^{natQ61R/+}*, *LSL-Kras^{comG12D/+}*, and *LSL-Kras^{comQ61R/+}* in a *Rosa26-CreERT2/+* background, injected with tamoxifen at 6 to 8 weeks of age and RNA was extracted from lungs seven days later. 2 *Rosa26-CreERT2/+* mice were treated similarly as a control (A). First strand cDNA synthesis and qRT-PCR of selected genes downstream ERK/MAPK pathway was performed using GoTaq 2-Step RT-qPCR kit (B,C). All measurements were normalized against Actin as the internal control using the $2^{-\Delta\Delta C_t}$ method. B and C are biological replicates.

A

B

C

D

E

F

Figure 2—figure supplement 1. Tumor phenotypes track with intrinsic oncogenic Kras signaling.

(A) Schematic of the experimental design.

(B) % of mice with at least one visible lung lesion.

(C) Representative H&E staining of lungs and (D) mean $\pm$ SD % tumor burden from microscopic analysis of two lung sections from two CC10-CreER/+;Kras+/+ mice 13 months after tamoxifen.

(E,F) Predominant proliferative lesions in lungs of CC10-CreER/+;Kras^{comG12D}/+ and CC10-CreER/+;Kras^{comQ61R}/+ differ.

Photomicrograph of lung section from (E) CC10-CreER/+;Kras^{comG12D}/+ mouse at 4 months showing multiple large pulmonary adenomas, and (F) CC10-CreER/+;Kras^{comQ61R}/+ mouse at 1 month showing extensive proliferation of airway epithelium into papillary projections of hyperplastic and dysplastic cells as well as pulmonary adenoma. H&E staining, x40 magnification.

Figure 3—figure supplement 1. Transcriptomes of each oncogenic *LSL-Kras* allele.

(A) Schematic of the experimental design.

(B) Volcano plot of the $\log_2$ fold-change versus p value of differentially expressed genes and (C) the false discovery rate (FDR) versus normalized enrichment score of the indicated GSEA hallmarks determined from RNA-seq analysis of the lungs of *Rosa26-CreERT2/+;LSL-Kras^{comQ61R/+}* versus *Rosa26-CreERT2/+;LSL-Kras^{natG12D/+}* mice seven days after tamoxifen injection.

(D) Principal component analysis of the transcriptome signatures of engineered alleles. Alleles with high quantitative signaling are highlighted with blue (*Kras^{comG12D/+}*) and pink shade (*Kras^{comQ61R/+}*), and alleles with and low signaling (*Kras^{nat}*) in green shade.

NatG12D/+ vs +/+

Figure 3—figure supplement 2. Transcriptome upon activating the *LSL-Kras^{natG12D}/+* allele.

Volcano plot of the $\log_2$ fold-change versus p value of differentially expressed genes from RNA-seq analysis of the lungs of *Rosa26-CreERT2/+;LSL-Kras^{natG12D}/+* versus *Rosa26-CreERT2/+;Kras+/+* mice seven days after tamoxifen injection.

NatQ61R/+ vs +/+

Figure 3—figure supplement 3. Transcriptome upon activation of the *LSL-Kras^{natQ61R}/+* allele.

Volcano plot of the $\log_2$ fold-change versus p value of differentially expressed genes from RNA-seq analysis of the lungs of *Rosa26-CreERT2/+;LSL-Kras^{natQ61R}/+* versus *Rosa26-CreERT2/+;Kras+/+* mice seven days after tamoxifen injection.

ComG12D/+ vs +/-

Figure 3—figure supplement 4. Transcriptome upon activating the *LSL-Kras^{comG12D}/+* allele.

Volcano plot of the $\log_2$ fold-change versus p value of differentially expressed genes from RNA-seq analysis of the lungs of *Rosa26-CreERT2/+;LSL-Kras^{comG12D}/+* versus *Rosa26-CreERT2/+;Kras+/+* mice seven days after tamoxifen injection.

Figure 3—figure supplement 5. Transcriptome upon activating the *LSL-Kras^{comQ61R/+}* allele.

Volcano plot of the $\log_2$ fold-change versus p value of differentially expressed genes from RNA-seq analysis of the lungs of *Rosa26-CreERT2/+;LSL-Kras^{comQ61R}/+* versus *Rosa26-CreERT2/+;Kras+/+* mice seven days after tamoxifen injection.

Figure 3—figure supplement 6

Figure 3—figure supplement 6. Transcriptome GSEA hallmarks upon activating the panel of *LSL-Kras* alleles.

Purple stars show alleles that show qualitative signaling dependent enrichment or depletion patterns of hallmarks. Only hallmarks with FDR less than 5% are shown. Dot size is adjusted according to RAS activity for better visualization.

Figure 3—figure supplement 7

Figure 3—figure supplement 7. Validation of signaling responses via qRT-PCR analysis of the selected genes.

(A) heatmap of the log₂ fold-change of selected genes from RNAseq analysis of the lungs of indicated *LSL-Kras* alleles in the *Rosa26-CreERT2*^{+/+} background in comparison to the wild type *Kras* allele seven days after tamoxifen injection.

(B,C) Validation of signaling responses via qRT-PCR analysis of the selected genes in A from pathways of TNF α Signaling via NF- κ B and Interferon γ Signaling (B), and EMT and Myogenesis (C). Two biological replicates.

Figure 4—figure supplement 1. Global activation of each engineered *LSL-Kras* allele using a ubiquitous Cre driver.

Schematic of the experimental design. Cohorts of three adult male and female *Rosa26-CreERT2/+*; *LSL-Kras^{natG12D}/+*, *Rosa26-CreERT2/+*; *LSL-Kras^{natQ61R}/+*, *Rosa26-CreERT2/+*; *LSL-Kras^{comG12D}/+*, and *Rosa26-CreERT2/+*; *LSL-Kras^{comQ61R}/+* mice, and as a control for normalization, *Rosa26-CreERT2/+*; *Kras^{+/+}* mice, were injected with tamoxifen and humanely euthanized at moribundity. Selected set of organs were fixed in formalin, sliced and embedded in paraffin, sectioned, and H&E stained.

Figure 4—figure supplement 2

Figure 4—figure supplement 2. Tissue-specific recombination rates of each oncogenic *LSL-Kras* allele upon global activation.

(A) Schematic of the experimental design. Two mice from each *LSL-Kras* genotype in a *Rosa26-CreERT2/+* background 7 days after tamoxifen injection were humanely euthanized, and their organs were removed. Genomic DNA was isolated and subjected to genotyping PCR. One mouse in *Rosa26-CreERT2/+;LSL-Kras^{comG12D/+}* background was used as a no tamoxifen control.

(B) Gel images from duplicate recombination analysis and (C) mean $\pm$ SEM of the ratio of the recombined *LSL-Kras* (LoxP recombined, 616 bp) to unaltered wild-type *Kras* (WT, 488 bp) allele products determined by PCR genotyping. Two technical replicates of two biological replicates. Full-length gel images are provided at **Figure 4—source data 1**.

(D,E) One-way Anova with Bonferroni's multiple comparisons test with a single pooled variance and a 95% CI shows variation in recombination rate across different alleles (D) compared to the *Kras^{natG12D/+}* allele or across organs (E) compared to the colon, with the exception of ovary, was not statistically significant (* $p < 0.1$ and *** $p < 0.001$).

Figure 4—figure supplement 3

Figure 4—figure supplement 3. Number of tumor types induced upon globally activating each oncogenic *LSL-Kras* allele.

Number of mice with the indicated number of different tumor types are shown (see **Supplementary File 3**).

Figure 4—figure supplement 4

Figure 4—figure supplement 4. Pairwise comparison of the survival curves of the engineered *LSL-Kras* alleles upon global activation in the *Rosa26-CreERT2/+* background mice after tamoxifen injection.

p values calculated by Log-Rank (Mantel-Cox) test of the pairwise comparisons of alleles (see **Supplementary File 2**).

Figure 4—figure supplement 5

Figure 4—figure supplement 5. Proliferative lesions induced upon activating each oncogenic *LSL-Kras* allele.

Examples of H&E stained sections from the indicated organs in each allele upon global activation at the moribundity endpoint.

Figure 4—figure supplement 6. Histopathological analysis of pulmonary lesions induced upon activating each of the oncogenic *LSL-Kras* alleles.

Mean $\pm$ SD of number of AHA and adenoma lesions per animal with stars denoting animals with extensive myeloid infiltration in the lungs (**A**), mean $\pm$ SD of peripheral lesions observed as percent surface area (**B**), and percent animals manifesting hyperplasia localized to bronchioles (**C**). p values calculated by two-way ANOVA multiple comparisons with Sidak testing (**A**) or one-way ANOVA multiple comparison with Tukey testing (**B**). ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$

Figure 4—figure supplement 7

Figure 4—figure supplement 7. The tumor landscape of *Rosa26-CreERT2/+;Kras^{rareG12D}/+* mice after tamoxifen injection.

Percent of the indicated organs with tumors from *Rosa26-CreERT2/+;LSL-Kras^{rareG12D}/+* mice (n=7) 20 months after tamoxifen injection.