Rare codons capacitate Kras-driven de novo tumorigenesis

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The KRAS gene is commonly mutated in human cancers, rendering the encoded small GTPase constitutively active and oncogenic. This gene has the unusual feature of being enriched for rare codons, which limit protein expression. Here, to determine the effect of the rare codon bias of the KRAS gene on de novo tumorigenesis, we introduced synonymous mutations that converted rare codons into common codons in exon 3 of the Kras gene in mice. Compared with control animals, mice with at least 1 copy of this Krasex3op allele had fewer tumors following carcinogen exposure, and this allele was mutated less often, with weaker oncogenic mutations in these tumors. This reduction in tumorigenesis was attributable to higher expression of the Krasex3op allele, which induced growth arrest when oncogenic and exhibited tumor-suppressive activity when not mutated. Together, our data indicate that the inherent rare codon bias of KRAS plays an integral role in tumorigenesis.

Introduction

The mammalian RAS family of small GTPases is composed of 3 genes that encode the proteins HRAS, NRAS, KRAS4A, and KRAS4B, the latter 2 being derived from alternatively spliced transcripts. RAS proteins transmit signals from surface receptors to intracellular signaling proteins. Upon receptor activation, guanine nucleotide exchange factors (GEFs) accelerate the exchange of GDP for GTP on RAS, which stabilizes an altered conformation that fosters binding and activation of effector proteins. In turn, GTPase-activating proteins (GAPs) increase the GTPase activity of RAS, returning the proteins to the inactive GDP-bound conformation. In a quarter or more of human cancers, one of the RAS genes has an activating or oncogenic mutation. These mutations typically occur at G12, G13, or Q61, with the frequency of each mutation in each RAS gene differing between cancers. While the underlying defect of mutations differs, each results in sustained GTP binding and RAS activation. Oncogenic RAS proteins chronically activate MAPK, PI3K, RAL, and other effector pathways to promote tumorigenesis (reviewed in refs. 1, 2).

The 4 RAS proteins share 79% amino acid sequence identity, with most of the variability residing in the last 25 amino acids that encode sites of different posttranslational modifications (3). The nucleotide sequences of the genes are, however, more divergent (4). Nucleotide differences often occur at the third, or wobble, position of the codon (5). In this regard, KRAS is preferentially encoded by A or T at wobble base pairs, HRAS by G or C, and NRAS by an intermediate mixture of nucleotides (4). Mammalian codons ending in A or T are less represented, or rare, in mammalian exomes (6). The functional significance of rare codons in transcripts of higher eukaryotes is poorly understood (5). Nevertheless, discordance in codon usage between genomes of different species can result in poor translation in heterologous systems (e.g., ref. 7). Rare codons can also reduce the efficiency of translation elongation, with functional consequences in other organisms (e.g., ref. 8). Similarly, we have previously demonstrated that rare codons impede KRAS translation, reducing protein expression relative to that of the other RAS isoforms (4). However, the consequences of altering codon usage within an endogenous gene have not been tested in mammalian organisms.

Oncogenic “driver” mutations in KRAS initiate the conversion of normal cells to a tumorigenic state (1). Paradoxically, oncogenic RAS, through activation of the MAPK pathway, can also induce senescent growth arrest in primary cells (9). One factor influencing this divergent response is the expression level of oncogenic RAS itself; with high expression inducing senescence and low expression promoting hyperplasia in vivo (10). The rare codon bias of KRAS has not been assessed with regard to tumor initiation, a process critically sensitive to RAS protein levels. Thus, we evaluated the effect of converting rare codons into common codons in the endogenous mouse Kras gene on in vivo carcinogenesis.

Results

Creation of mice with a codon-altered Kras allele. To investigate the influence of rare codons in Kras in vivo, a knock-in approach was designed to minimally perturb the murine Kras gene while maximally altering codon bias. Specifically, converting rare codons into common codons in exon 3 increased ectopic KRAS protein expression more, for reasons that remain to be elucidated, than did similar alternations to exons 1 or 2 (Figure 1A). Thus, we designed a knock-in construct to introduce 33 synonymous mutations into murine Kras exon 3 that optimized (ex3op) 27 rare codons by converting them into more common codons, thereby increasing common codon usage (Figure 1, B–D, and Supplemental Figure 1; supplemental material available online with this article;
doi:10.1172/JCI77627DS1). Heterozygous Kras<sup>ex3op(neo)</sup> mice, identified by genotyping PCR that yielded the expected 388-bp product (Figure 1, D and E), were crossed with C57BL6/J-CMV-Cre or, to maintain a 129 background, with 129Sv-PRM-Cre transgenic mice to induce Cre-mediated recombination between loxP sites and delete the neo cassette. Successful excision of the neo cassette in the resulting offspring was identified by PCR amplification of genomic DNA, yielding the expected 504-bp product (Figure 1, D and E).

To determine the impact of converting rare codons into common codons on endogenous KRAS mRNA and protein levels, SV40-immortalized mouse embryonic fibroblasts (MEFs) were generated from littermates homozygous for the ex3op or native Kras alleles. Semiquantitative reverse transcription PCR (RT-PCR) revealed no overt difference in total Kras mRNA expression levels between Kras<sup>ex3op/</sup>ex3op and Kras<sup>nat/</sup>nat MEFs. We also observed no obvious differences in the levels of Kras<sup>4a</sup> and Kras<sup>4b</sup> splice forms (Figure 1F), an important consideration, given that synonymous mutations can alter splicing of oncogenes (11) and that both splice forms of Kras are known to contribute to tumorigenesis (12, 13). While mRNA levels remained unchanged, immunoblot analysis revealed roughly 2-fold more endogenous KRAS protein in Kras<sup>ex3op/</sup>ex3op MEFs compared with that detected in Kras<sup>nat/</sup>nat MEFs (Figure 1G). Finally, although we observed considerable variability between mice of the same genotype (data not shown), immunoblot comparisons of 16 individual age-matched pairs revealed, on average, more endogenous KRAS protein in lung tissue from Kras<sup>ex3op/</sup>ex3op mice compared with that from Kras<sup>nat/</sup>nat mice (Figure 1H). Thus, 33 synonymous point mutations in exon 3 of the murine Kras gene resulting in the conversion of 27 rare codons to common codons increased the average amount of KRAS protein detected in the tested cells and tissues.

Kras<sup>ex3op/</sup>ex3op mice have no overt developmental phenotype. To investigate the developmental consequences of changing Kras codon bias, Kras<sup>ex3op/</sup>nat mice were crossed and the frequency of offspring with each of 3 possible genotypes assessed. Of 295 offspring, we observed no difference from the expected Mendelian ratios of Kras<sup>ex3op/</sup>ex3op, Kras<sup>ex3op/</sup>nat, and Kras<sup>ex3op/</sup>ex3op mice (Supplemental Table 1). The animals also had no obvious physical or reproductive abnormalities (data not shown), and we noted no significant differences in weight between adult Kras<sup>ex3op/</sup>nat and Kras<sup>ex3op/</sup>ex3op mice, regardless of age or strain background (Supplemental Figure 2). To investigate the Kras<sup>ex3op</sup> allele in a background sensitive to decreased Kras gene dosage, we assessed the postnatal development of chylous ascites. Chylous ascites arises in Kras<sup>+/−</sup> mice on either an Hras<sup>−/−</sup> or Nras<sup>−/−</sup> background, and the incidence of this phenotype is inversely correlated with the number of functional Ras alleles (14). Thus, Kras<sup>ex3op/</sup>nat Nras<sup>−/−</sup> Kras<sup>+/−</sup> mice were crossed and 121 offspring evaluated. Again, we observed no significant differences from the expected Mendelian ratios of offspring, and the incidence of chylous ascites was not significantly different between the 3 Kras genotypes (Supplemental Tables 2 and 3). Hence, the Kras<sup>ex3op</sup> allele appears developmentally equivalent to the native Kras allele.

Kras<sup>ex3op/</sup>nat mice develop fewer lung tumors following carcinogen exposure. To investigate the effect of the Kras<sup>ex3op</sup> allele on tumorigenesis, mice were administered the chemical carcinogen urethane to induce lung adenomas characterized by Q61L or R oncogenic mutations in the Kras gene (15). To populate mice for this study, we crossed Kras<sup>ex3op(neo)/</sup>nat mice with Kras<sup>nat/</sup>nat mice containing the CMV-Cre transgene to excise the neo cassette used in the generation of the Kras<sup>ex3op(neo)</sup> allele (Figure 2A). We injected cohorts of Kras<sup>ex3op/</sup>nat CMV-Cre and Kras<sup>nat/</sup>nat CMV-Cre littermates with urethane at 6 to 8 weeks of age. Eleven to 12 months later, when tumor incidence was expected to be near 100% (16), the surviving mice were euthanized and their lungs removed to assess tumor number and size. We calculated the tumor burden (defined as the sum of tumor diameters) from these values. Kras<sup>ex3op/</sup>nat CMV-Cre mice exhibited a greater than 2-fold decrease in tumor burden compared with that observed in their Kras<sup>nat/</sup>nat CMV-Cre littermates (Figure 2B). We observed no lung lesions in untreated mice of either genotype (data not shown). To ascertain the reproducibility of this effect at an earlier time point (9 months), we administered urethane to another independent cohort of Kras<sup>ex3op/</sup>nat CMV-Cre and Kras<sup>nat/</sup>nat CMV-Cre littermates. Mice carrying the Kras<sup>ex3op</sup> allele again exhibited a greater than 2-fold reduction in tumor burden (Figure 2C). We confirmed the excision of the neo cassette in tested tumors arising in Kras<sup>ex3op/</sup>nat CMV-Cre mice and observed no obvious differences in tumor histology between cohorts (Supplemental Figure 3, A and B). Finally, to determine whether these results were reproducible between targeting events, Kras<sup>ex3op/</sup>nat mice generated from an independently targeted embryonic stem (ES) cell clone were crossed with Kras<sup>nat/</sup>nat CMV-Cre mice. We injected the resultant Kras<sup>ex3op/</sup>nat CMV-Cre and Kras<sup>nat/</sup>nat CMV-Cre littermates with urethane and analyzed them 9 months later for lung tumor burden. Again, we found that Kras<sup>ex3op/</sup>nat CMV-Cre mice exhibited a nearly 4-fold reduction in tumor burden (Figure 2D). In all 3 experiments, the reduction in tumor burden was primarily a consequence of decreased tumor multiplicity and not tumor size, suggesting fewer successful tumor initiation events (Supplemental Figure 3, C-E). We also found no detectable difference between urethane-induced tumors arising in Kras<sup>ex3op/</sup>nat CMV-Cre and Kras<sup>nat/</sup>nat CMV-Cre mice in terms of Ki67 immunoreactivity, a marker of cell proliferation; TUNEL activity, a marker of cell death; or Nqo1 immunoreactivity, a protein reported to be part of the NRF2 antioxidant and cellular detoxification program induced by oncocgenic Kras during pancreatic cancer (17), supportive of the Kras<sup>ex3op</sup> allele influencing early tumorigenesis (Supplemental Figure 3, F-H). Collectively, these data suggest that converting rare codons to common codons in Kras reduces the initiation of tumors induced by the carcinogen urethane.

Reduced Kras<sup>G12D</sup>-driven tumorigenesis in the presence of a nononcogenic Kras<sup>ex3op</sup> allele. The observed reduction in tumor burden in Kras<sup>ex3op/</sup>nat mice could be an effect of changing codon bias in the oncogenic or nononcogenic allele. Loss of 1 Kras allele can substantially increase sensitivity to urethane, suggesting that the nononcogenic (WT) Kras allele suppresses the tumorigenic activity of the oncogenic Kras allele (18). As such, increasing expression of the nononcogenic Kras allele, in this case by converting rare codons into common codons, could be expected to reduce urethane carcinogenesis (19). To directly test this possibility, we crossed B6 129S-Kras<sup>ex3op/</sup>nat mice with Kras<sup>LSL-G12D/</sup>nat mice from a mixed background, which harbor a Cre-inducible Kras<sup>LSL-G12D</sup>
Figure 1. Generation and characterization of the Kras<sub>ex3op</sub> allele. (A) Immunoblot reveals changing rare codons to common codons in exon 3 increases ectopic KRAS expression (1 of 2 experiments using cells independently transduced with expression vectors). (B) Position of synonymous mutations (red) in Kras<sub>ex3op</sub> aligned to Kras<sub>nat</sub> within exon 3. Lowercase letters denote nucleotides from exon 2. (C) Sliding window of codon usage index demonstrating the relative codon usage of the mouse Ras genes including the engineered enrichment of common codons in exon 3 of Kras<sub>ex3op</sub> (red) relative to Kras<sub>nat</sub> (gray). Theoretical Kras transcripts encoded by all common (red dotted line) or all rare (black dotted line) are plotted for reference. (D) Targeting strategy to change codon usage in exon 3 of Kras. Arrows indicate PCR primers used for genotyping. (E) Representative genotyping from mice with Kras<sub>nat</sub> and/or Kras<sub>ex3op</sub> alleles used in all genotyping experiments. (F) Semiquantitative RT-PCR analysis revealed similar levels of Kras splice forms 4a and 4b in MEFs isolated from Kras<sub>ex3op/ex3op</sub> versus those from Kras<sub>nat/nat</sub> mice (1 of 2 experiments using independently derived MEFs). (G) Representative example and quantification (mean ± SEM) of immunoblots revealed higher levels of KRAS protein in lysates of MEFs isolated from Kras<sub>ex3op/ex3op</sub> mice than levels detected in Kras<sub>nat/nat</sub> mice (1 of 2 experiments using independently-derived MEFs). (H) Representative example and quantification (mean ± SEM) of immunoblots revealed higher levels of KRAS protein in total lung lysates isolated from Kras<sub>ex3op/ex3op</sub> mice than those detected in Kras<sub>nat/nat</sub> mice. Comparisons were made using lysates from a total of 16 Kras<sub>ex3op/ex3op</sub> versus 16 Kras<sub>nat/nat</sub> mice.
Kras<sup>ex3op</sup> or 129-Kras<sup>nat</sup> allele from 1 parent and the remaining BL6-Kras<sup>nat</sup> allele inherited from the other parent (Figure 2A and Supplemental Figure 5). RNA was isolated from tumors arising from the aforementioned Kras<sup>ex3op</sup>/nat CMV-Cre and Kras<sup>nat</sup>/nat CMV-Cre littermates injected with urethane from the 2 different founder lines (Figure 2, B and D) and RT-PCR amplified with primers specific for Kras (Figure 4A). The products were cloned into a plasmid vector, and individual inserts were sequenced. We sequenced 8–12 independent inserts from each tumor to identify (a) the strain (129 or BL6) based on SNPs, (b) the type of Kras allele (nat or ex3op) based on the exon 3 sequence, and (c) the presence of oncogenic mutations (Supplemental Table 4). As expected (15), we found that almost all tumors from Kras<sup>nat</sup>/nat CMV-Cre mice carried an oncogenic Q61L or R (Q61L/R) mutation. Consistent with the 129 strain being more susceptible to urethane mutagenesis than the BL6 strain (18), all oncogenic Kras mutations detected in tumors from these mice occurred in the 129-Kras<sup>nat</sup> allele. In contrast, the percentage of tumors from Kras<sup>ex3op</sup>/nat CMV-Cre mice with a Kras oncogenic mutation occurring in the 129-Kras<sup>ex3op</sup> allele was reduced by half. Moreover, instead of canonical Q61L/R mutations, we detected G12D mutations, which are rarely detected following urethane administration (21), in the 129-Kras<sup>ex3op</sup> allele (Figure 4B). We observed no significant differences in the mutation spectrum between the 2 founder lines (Supplemental Table 4).

To independently validate these results using a more sensitive assay, Kras mRNA from tumors of another cohort of mice injected with urethane was analyzed. Cohorts of Kras<sup>LSL-G12D/ex3op</sup> and Kras<sup>LSL-G12D/nat</sup> littermates were administered adenovirus encoding Cre recombinase (AdCre) via intranasal inhalation to activate the oncogenic Kras<sup>LSL-G12D</sup> allele in lung epithelium and initiate lung tumorigenesis (Figure 3A). Four and 6 months later, mice were euthanized, the lungs removed, and tumor burden assessed. We found that AdCre-treated Kras<sup>LSL-G12D/ex3op</sup> mice exhibited at least a 2-fold reduction in tumor burden compared with that seen in control Kras<sup>LSL-G12D/nat</sup> mice at both time points (Figure 3, B and C). At the 4-month time point, this difference was primarily due to a reduction in tumor incidence, similar to what was observed in the mice injected with urethane, but by 6 months, decreased lesion size was evident (Supplemental Figure 4, A and B). No overt differences in tumor histology were apparent between genotypes (Supplemental Figure 4C). With the caveat that the Kras alleles were not congenic (19), the Kras<sup>LSL-G12D</sup> allele more potently suppresses oncogenic Kras-driven lung tumor formation.

The Kras<sup>ex3op</sup> allele is less frequently mutated in urethane-induced lung tumors. We next determined the difference in frequency and type of oncogenic mutations occurring in the Kras<sup>ex3op</sup> and Kras<sup>nat</sup> alleles inherited from the same parent. There are a number of SNPs in the Kras gene that differ between the 129S6/SvEvTac (termed 129 hereafter) background on which Kras<sup>ex3op</sup> mice were generated and the C57BL/6/J (termed BL6 hereafter) strain that carries the CMV-Cre transgene used to excise the neo cassette (Figure 4A and Supplemental Figure 5). We used these SNPs to identify the 129-Kras<sup>ex3op</sup> or 129-Kras<sup>nat</sup> allele from 1 parent and the remaining BL6-Kras<sup>nat</sup> allele inherited from the other parent (Figure 2A and Supplemental Figure 5). RNA was isolated from tumors arising from the aforementioned Kras<sup>ex3op/nat</sup> CMV-Cre and Kras<sup>nat/nat</sup> CMV-Cre littermates injected with urethane from the 2 different founder lines (Figure 2, B and D) and RT-PCR amplified with primers specific for Kras (Figure 4A). The products were cloned into a plasmid vector, and individual inserts were sequenced. We sequenced 8–12 independent inserts from each tumor to identify (a) the strain (129 or BL6) based on SNPs, (b) the type of Kras allele (nat or ex3op) based on the exon 3 sequence, and (c) the presence of oncogenic mutations (Supplemental Table 4). As expected (15), we found that almost all tumors from Kras<sup>ex3op/nat</sup> CMV-Cre mice carried an oncogenic Q61L or R (Q61L/R) mutation. Consistent with the 129 strain being more susceptible to urethane mutagenesis than the BL6 strain (18), all oncogenic Kras mutations detected in tumors from these mice occurred in the 129-Kras<sup>ex3op</sup> allele. In contrast, the percentage of tumors from Kras<sup>ex3op/nat</sup> CMV-Cre mice with a Kras oncogenic mutation occurring in the 129-Kras<sup>ex3op</sup> allele was reduced by half. Moreover, instead of canonical Q61L/R mutations, we detected G12D mutations, which are rarely detected following urethane administration (21), in the 129-Kras<sup>ex3op</sup> allele (Figure 4B). We observed no significant differences in the mutation spectrum between the 2 founder lines (Supplemental Table 4).

To independently validate these results using a more sensitive assay, Kras mRNA from tumors of another cohort of mice injected
with urethane (Figure 2C) was RT-PCR amplified and subjected to massively parallel sequencing using the Ion Torrent PGM platform. Based on a minimum of 100 independent sequence reads of each allele, we detected a Q61R mutation in the 129-Krasnat allele in the majority (57%) of tumors from Krasnat/nat CMV-Cre mice. Again, we found that the 129-Krasex3op allele was mutated in only one-third of the tumors arising in Krasex3op/nat CMV-Cre mice, and only G12D mutations were recovered (Figure 4C and Supplemental Table 5). Thus, we detected fewer and different oncogenic mutations in the Krasex3op allele of urethane-induced tumors.

Dose-dependent changes are observed with the Krasex3op allele. The above approach permitted a detailed analysis of the relative mutation frequency of the Krasex3op allele versus that of the Krasnat allele inherited from the same parental strain, but required the carcinogen to be administered to F1 progeny of a mixed BL6/129 background. This precluded treatment of mice homozygous for the Krasnat allele. Thus, we repeated the analysis in a 129 background to assess all possible Kras genotypes. Specifically, we crossed 129-Krasex3op(neo)/nat PRM-Cre mice with a 129S/Sv-PRM-Cre line that expresses Cre recombinase in the male germline. Male 129-Krasex3op(neo)/nat PRM-Cre offspring were crossed with 129 females to generate progeny with confirmed Krasex3op allele (Supplemental Figures 3 and 6). KRAS protein levels were elevated in the Krasex3op(ex3op) allele and occurred at high levels of transgenic HRASG12V have been shown to induce widespread senescence in the mouse mammary gland in vivo (10). Furthermore, converting rare codons into common codons in KRAS suppressed cell proliferation of normal cells (4). Thus, higher protein levels from the Krasex3op allele may increase the chance that an oncogenic mutation will result in growth arrest, accounting for the reduced number of tumors in Krasex3op(ex3op) mice injected with urethane.

To assess the response of normal cells to oncogenic codon-altered Kras, each of the oncogenic Kras mutations recovered from urethane-induced lung tumors, namely, G12V, G12D, Q61L, and Q61R, were introduced into FLAG epitope–tagged nat or ex3op Kras cDNAs. The human lung fibroblast cell line IMR90, which undergoes growth arrest upon expression of oncoenic HRAS (23), was stably infected with retroviruses encoding no oncogene as a negative control (empty), FLAG-HRASG12V as a positive control, or either FLAG-Krasnat or FLAG-Krasex3op with 1 of the 4

**Figure 3. Increased resistance of Krasex3op mice to lung tumorigenesis driven by Krasex3op.** (A) Strategy to generate KrasLSL–G12D/ex3op and KrasLSL–G12D/nat littermates for analysis of tumor burden. (B and C) Reduction of tumor burden (mean ± SEM) in KrasLSL–G12D/ex3op versus KrasLSL–G12D/nat mice (B) 4 months (n ≥8) and (C) 6 months (n ≥9) after AdCre administration. *P < 0.05 by unpaired, 2-tailed t test.
aforementioned oncogenic mutations. Immunoblot analysis revealed the expected robust expression of FLAG-HRAS^{G12V} (4) and higher expression of FLAG-KRAS in cells transduced with oncogenic FLAG-Kras^{ex3op} transgenes compared with those transduced with the FLAG-Kras^{nat} counterparts with the same oncogenic mutations. In agreement with these findings, we found that phosphorylated (T202/4) ERK1/2 (p-ERK), a measure of oncogenic RAS activation of the MAPK pathway, was also slightly elevated in cells transduced with FLAG-Kras^{ex3op} compared with those transduced with FLAG-Kras^{nat} oncogenes (Figure 6A). Thus, conversion of rare codons into common ones increases ectopic KRAS protein expression and downstream MAPK signaling.

A comparison between Q61L/R and G12V/D mutations in the FLAG-KRAS protein revealed that expression levels of the former mutant were unexpectedly higher and correspondingly stimulated the MAPK pathway to a greater degree, as assessed by p-ERK levels. The difference between these mutants was observed in both the nat and ex3op versions of FLAG-KRAS, although the effect was more pronounced in the FLAG-Kras^{ex3op} lines (Figure 6A). These mutations are structurally and biochemically distinct. Q61 mutations alter a coordinating catalytic amino acid (24, 25), while G12 mutations sterically inhibit the arrangement of GAP and RAS-Q61 residues critical for GTP hydrolysis (26). In general agreement with this, pull-down of RAS with the RAF1 RAS–binding domain (RBD) revealed higher levels of GTP-bound KRAS from cells transduced with FLAG-Kras^{ex3op} than were detected from matched FLAG-Kras^{nat}–transduced cells and, at least in the case of Q61R, higher levels of GTP-bound KRAS than were detected with G12V/D mutants (Supplemental Figure 7). Thus, oncogenic mutations in Kras^{ex3op} and Q61L/R mutations are comparatively more potent than oncogenic mutations in Kras^{nat} and G12V/D mutations, respectively, as assessed by KRAS-GTP and p-ERK levels. These results suggest that the most potent oncogenic combination is that of Q61L/R mutations in the Kras^{ex3op} codon–optimized version of Kras.

Oncogenic Kras^{ex3op} suppresses cell proliferation. Growth arrest in response to oncogenic stress induced by oncogenic RAS is largely attributed to stimulation of the MAPK pathway (9). Immunoblot analysis revealed that expression of FLAG-Kras^{ex3op} with Q61L/R oncogenic mutations led to the highest level of MAPK activation (Figure 6A). Consistent with this, only expression of FLAG-Kras^{ex3op} with a Q61L/R oncogenic mutation induced upregulation of p16 (Figure 6A), a marker of cellular senescence (23). Q61L/R mutations in Kras^{ex3op} were also very rarely recovered from urethane-induced tumors. One explanation for the observed reduction in tumor burden and mutations recovered from the Kras^{ex3op} allele is that Q61L/R mutations, in the context of a codon-optimized exon 3 transcript, result in higher levels of activated KRAS that mediate growth arrest and hence are rarely detected.

To explore this hypothesis, the above primary lung fibroblasts were stably transduced with HRAS^{G12V} and the different mutant versions of Kras^{nat} and Kras^{ex3op} (Figure 6A). We calculated cell doublings when the vector control cells approached confluence. As expected (4, 23), cells expressing HRAS^{G12V} exhibited a pronounced

**Figure 4. Detection of fewer and different mutations in the Kras^{ex3op} allele from tumors of mice treated with urethane.**

(A) Amplification strategy for Kras RT-PCR including location of SNPs used for strain identification. Green arrows indicate primers for Sanger sequencing; blue arrows indicate primers for Ion Torrent sequencing. (B and C) Sequencing by the (B) Sanger and (C) Ion Torrent methods of Kras cDNAs from the indicated number (n) of tumors arising in the indicated urethane-treated cohorts revealed fewer and different mutations in the Kras^{ex3op} allele. Pie charts are sized to reflect differences in overall tumor multiplicity for each genotype.
growth arrest compared with that observed in vector control cells. Consistent with the immunoblot analysis, Q61L/R mutations in KrasNat suppressed cell proliferation more than did G12V/D mutations. We observed a similar trend with KrasEx3op oncogenes. Additionally, expression of KrasEx3op with 3 of the 4 oncogenic mutants suppressed proliferation to a greater degree than did corresponding KrasNat oncogenes, although the difference only reached statistical significance in 1 comparison (Figure 6B). To independently assess whether the differences observed in growth arrest were attributable to altered codon bias, we synthesized completely artificial Kras cDNAs using all common codons (KrasCommon) or all rare codons (KrasRare). We found that expressing KrasCommon in either the G12D- or Q61R-mutant configuration suppressed cell proliferation to a greater degree than did KrasRare with the same oncogenic mutations (Figure 6C). Thus, Kras oncogenes enriched by common codons more potently suppress proliferation of primary cells.

A Cdkn2a-null background blunts KrasEx3op inhibition of lung tumor initiation. Based on the above results, oncogenic mutations in the KrasEx3op allele may lead to growth arrest, thereby accounting for the reduced tumor burden in KrasEx3op mice injected with urethane. Expression of oncogenic Kras in mouse lungs induces p16INK4A expression and a senescent growth arrest (27). Disruption of the Cdkn2a locus encoding p16INK4A and p19ARF is known to partially suppress Ras oncogene–induced senescence (28–30) and
permits sustained MAPK signaling and tumor progression in an oncogenic Kras-driven mouse model of lung cancer (31). Hence, suppressing the ability of normal cells to respond to oncogenic stress vis-à-vis disruption of the Cdkn2a locus may permit oncogenic mutations in the Krasex3op allele to be better tolerated.

To test the effect of a Cdkn2a-null background on urethane-induced lung tumorigenesis in mice with nat or ex3op Kras alleles, Krasex3op/nat Cdkn2a +/– mice were crossed to populate cohorts of Krasnat/nat, Krasex3op/nat, and Kras ex3op/ex3op littermates that were either Cdkn2a–/– or Cdkn2a+/+(Figure 7A). Mice were administered urethane and assessed for lung tumor burden 2.5–3 months later for Cdkn2a–/– cohorts and 4 months later for Cdkn2a+/+ cohorts. Early assessment of lung lesions was necessary in the Cdkn2a–/– cohorts due to accelerated moribundity not associated with lung tumor burden (data not shown). While the overall incidence of lung lesions at this early time point was low, we found no statistical difference in tumor number, size, or burden between the 3 Kras genotypes (Figure 7, B–D). In fact, there instead appeared to be a trend toward increased tumor size in Krasex3op/ex3op mice, which we did not observe in Cdkn2a+/+ mice (Figure 7D and Supplemental Figure 8C). These tumors were the result of urethane carcinogenesis, as no lung lesions were detected in any untreated Cdkn2a–/– cohorts at this time point (data not shown).

Furthermore, we found that matched Cdkn2a+/+ littermates retained significant differences in tumor burden between Kras genotypes, as observed in previous experiments (Supplemental Figure 8). Thus, a Cdkn2a+/+ background dampens the differences in tumor initiation between Krasnat/nat, Kras ex3op/nat, and Krasnat/nat mice injected with urethane.

Discussion

It is well appreciated that protein expression is affected by codon bias in heterologous systems (7). There is also emerging evidence that rare codons within mammalian genes can have biochemical or cellular consequences (4, 32–35). We now show that permits sustained MAPK signaling and tumor progression in an oncogenic Kras-driven mouse model of lung cancer (31). Hence, suppressing the ability of normal cells to respond to oncogenic stress vis-à-vis disruption of the Cdkn2a locus may permit oncogenic mutations in the Krasex3op allele to be better tolerated.

To test the effect of a Cdkn2a-null background on urethane-induced lung tumorigenesis in mice with nat or ex3op Kras alleles, Krasex3op/nat Cdkn2a +/– mice were crossed to populate cohorts of Krasnat/nat, Krasex3op/nat, and Kras ex3op/ex3op littermates that were either Cdkn2a–/– or Cdkn2a+/+(Figure 7A). Mice were administered urethane and assessed for lung tumor burden 2.5–3 months later for Cdkn2a–/– cohorts and 4 months later for Cdkn2a+/+ cohorts. Early assessment of lung lesions was necessary in the Cdkn2a–/– cohorts due to accelerated moribundity not associated with lung tumor burden (data not shown). While the overall incidence of lung lesions at this early time point was low, we found no statistical difference in tumor number, size, or burden between the 3 Kras genotypes (Figure 7, B–D). In fact, there instead appeared to be a trend toward increased tumor size in Krasex3op/ex3op mice, which we did not observe in Cdkn2a+/+ mice (Figure 7D and Supplemental Figure 8C). These tumors were the result of urethane carcinogenesis, as no lung lesions were detected in any untreated Cdkn2a–/– cohorts at this time point (data not shown).

Furthermore, we found that matched Cdkn2a+/+ littermates retained significant differences in tumor burden between Kras genotypes, as observed in previous experiments (Supplemental Figure 8). Thus, a Cdkn2a+/+ background dampens the differences in tumor initiation between Krasnat/nat, Kras ex3op/nat, and Krasnat/nat mice injected with urethane.
altering rare codons. However, the parsimonious nature of changes to the \( Kras \) locus and the consistent phenotype of the \( Kras^{ex3op} \) oncogenes in an independent, cell-based assay are supportive of a model in which oncogenic mutations in \( Kras^{ex3op} \) allele are selected against due to higher protein expression inducing growth arrest instead of proliferation. Extending these results one step further, we speculate that poor expression imposed by rare codons may increase the likelihood that an oncogenic mutation in \( KRAS \) will lead to proliferation instead of growth arrest, which may account for the high frequency of mutations in \( KRAS \) compared with those occurring in the other \( RAS \) genes in human cancers (1, 2). Paradoxically, rare codons hamper the oncogenic activity of \( KRAS \) in cells resistant to oncogene-induced stress (4). As such, we speculate that the rare codon bias of oncogenic \( KRAS \) poses a barrier to more malignant phenotypes once cells overcome oncogene-induced stress, which may lead to the selection of mechanisms to increase RAS signaling.

With regard to the nononcogenic \( Kras \) allele, decreased \( Kras \) gene dosage increases sensitivity to urethane carcinogenesis, indicative of a tumor-suppressive role for the WT \( Kras \) allele (18). In agreement with this finding, we now show that the converse is true, namely, that converting rare codons into common codons in the nononcogenic \( Kras^{ex3op} \) allele also yields higher protein levels and reduced tumor burden in mice injected with urethane. Admittedly, these effects could be the product of other features intrinsic to the \( Kras \) locus altered by the targeting strategy (12, 13, 19, 36) or other unknown effects of altering rare codons. However, the parsimonious nature of changes to the \( Kras \) locus and the consistent phenotype of the \( Kras^{ex3op} \) oncogenes in an independent, cell-based assay are supportive of a model in which oncogenic mutations in \( Kras^{ex3op} \) allele are selected against due to higher protein expression inducing growth arrest instead of proliferation. Extending these results one step further, we speculate that poor expression imposed by rare codons may increase the likelihood that an oncogenic mutation in \( KRAS \) will lead to proliferation instead of growth arrest, which may account for the high frequency of mutations in \( KRAS \) compared with those occurring in the other \( RAS \) genes in human cancers (1, 2). Paradoxically, rare codons hamper the oncogenic activity of \( KRAS \) in cells resistant to oncogene-induced stress (4). As such, we speculate that the rare codon bias of oncogenic \( KRAS \) poses a barrier to more malignant phenotypes once cells overcome oncogene-induced stress, which may lead to the selection of mechanisms to increase RAS signaling.

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although this remains to be tested. As such, the contribution of WT RAS proteins to signaling may be tumor suppressive during tumor initiation, at least in some settings, but tumor promoting once tumor cells become insensitive to oncogene-induced stress.

The observed shift from Q61L/R to G12V/D mutations in the Kras<sup>rare</sup> allele in the tumors of some strain backgrounds after urethane administration may also be related to oncogenic potency. Q61L/R mutations appeared more active than G12V/D mutations in vitro and resulted in more potent growth arrest of primary lung fibroblasts. In fact, Q61L/R mutations in Kras<sup>rare</sup> were the only Kras oncogenes that induced detectable p16 expression. These mutations were very rarely detected in the Kras<sup>common</sup> allele of lung tumors. This raises the intriguing possibility that the ostensibly more potent Q61L/R mutations were selected against in the more highly expressed Kras<sup>common</sup> allele, perhaps in favor of the apparently weaker G12V/D mutations, which may evade a growth-arrest response. On the other hand, G12V/D mutations are normally rarely detected in the Kras allele of lung tumors developing after administration of urethane. Thus, these mutations may be too weak to promote tumorigenesis from a native Kras allele, hence the common detection of Q61L/R mutations in urethane-induced lung tumors. In agreement with these findings, it was recently reported that NRAS<sup>Q61L/R</sup>, but not NRAS<sup>G12D</sup>, promotes melanoma-lung tumors. Among vertebrate Kras transcripts, these lysines are always encoded by a mixture of the more rare (AAA) and more common (AAG) codons, and more than 5 adenines are not observed. Hence, alternating AAA and AAG codons were used in both the Kras<sup>common</sup> and Kras<sup>rare</sup> cDNAs.

**Methods**

**Generation of codon-altered Kras exon 3 knock-in (Kras<sup>ex3op</sup>) mice.** A knock-in construct was created using long and short arms of homology from a 129S7/AB2.2 genomic mouse BAC and a synthetic exon 3, in which all rare codons were converted into 1 of the 2 most commonly used codons in the mouse genome, provided they were not located in annotated or likely sites that would affect splicing of the gene. Targeting of the Kras allele in 129S6-Taconic ES cells and blastocyst injection were performed using standard procedures.

**Codon usage plots.** The codon usage index was calculated according to the method described for the codon adaptation index (44), but with the relative codon frequency derived from codon usage in the mouse exome (6). Values were calculated for sliding windows of 25 codons across each transcript. A theoretical Kras transcript encoded by all common (red dotted line) or all rare (black dotted line) codons were plotted for reference (Figure 1C).

**Plasmids.** FLAG-tagged RAS constructs were subcloned into pBabePuro for the generation of retrovirus. FLAG-tagged human HRAS and KRAS constructs were described previously (37). FLAG-tagged chimeric KRAS with exon substitutions were constructed by replacing the indicated exons with HRAS codons. FLAG-tagged mouse Kras<sup>4b</sup> cDNAs were subcloned from mouse tumors. Oncogenes not recovered from tumor samples were created using site-directed mutagenesis. FLAG-Kras<sup>common</sup> and FLAG-Kras<sup>rare</sup> were synthesized by GeneArt Gene synthesis (Invitrogen).

**Design of synthetic Kras<sup>common</sup> and Kras<sup>rare</sup> sequences.** Selection of common and rare codons for Kras<sup>common</sup> and Kras<sup>rare</sup> cDNAs was done on the basis of annotated codon usage in the mouse exome (6). These cDNAs used entirely rare or common codons with one minor exception. The poly-lysine stretch in the Kras<sup>4b</sup> C terminus (beginning at codon 176) was not fully optimized due to concerns about artifacts resulting from 18 sequential adenines in the rare cDNA (49). Among vertebrate Kras transcripts, these lysines are always encoded by a mixture of the more rare (AAA) and more common (AAG) codons, and more than 5 adenines are not observed. Hence, alternating AAA and AAG codons were used in both the Kras<sup>common</sup> and Kras<sup>rare</sup> cDNAs.

**Semiquantitative Kras RT-PCR.** RNA was isolated from MEFs using RNA-Bee (Fisher Scientific). Reverse transcription reactions were primed with Oligo(dT) (Invitrogen) using M-MuLV Reverse Transcriptase (New England Biolabs) with the addition of RNaseOUT (Invitrogen). PCR amplification was performed using Platinum Taq (Invitrogen) polymerase, with the supplied buffer used at 0.7 times the suggested final concentration, 1 mM dNTPs, 2 mM MgCl<sub>2</sub>, and 0.4 μM forward and reverse primers. Primer sequences are detailed in Supplemental Table 7. Reactions were run using the standard PCR conditions of denaturation at 94°C for 30 seconds, annealing at 50°C for 30 seconds, and elongation at 72°C for 2 minutes. Cycle numbers are indicated on the corresponding figure. Bands were resolved using 2% agarose gel electrophoresis with ethidium bromide staining. Results are representative of comparisons between Kras<sup>mut/+</sup> and Kras<sup>ex3op/ex3op</sup> littersome MEFs derived from 2 litters.

**Protein analysis.** Whole-cell lysates were prepared from pelleted cultured cells (see below) stored at −80°C until lysed. Lysates from whole lung tissue were prepared by snap freezing, followed by mortar and pestle tissue disruption under liquid nitrogen. RAS-GTP pull-down assays were conducted as previously described using 1 mg total protein (46). Equal protein levels were resolved and immunoblotted with antibodies against the indicated proteins (Figure 1A, A, G, and H, Figure 6A, and Supplemental Figure 7), as previously described (47). Briefly, SDS-PAGE was followed by transfer to a PVDF membrane (Millipore), blocking in 5% BSA (Sigma-Aldrich), and immunoblotting with one of the following antibodies diluted, as indicated, in 5% BSA: αKRAS (1:50, SC-30; Santa Cruz Biotechnology Inc.); α-tubulin (1:1,000, TUB21; Sigma-Aldrich); β-actin (1:25,000, AC-74; Sigma-Aldrich); αFLAG (1:1,000, M2; Sigma-Aldrich); p(Thr202/204) ERK1/2 (1:200, E10; Cell Signaling Technology); or αβ-actin (1:200, G175-405; BD Biosciences). Signal intensity quantified with ImageJ software (NIH) (48) was normalized to loading controls and compiled from at least 2 immunoblots. Endogenous KRAS is reported relative to Kras<sup>ex3op</sup> samples, FLAG-tagged oncogenes are reported relative to HRAS<sup>mut/+</sup>, and p-ERK1/2 is reported relative to empty control.

**Cell culture.** MEFs were isolated from E13.5 mouse embryos, as previously described (23), and stably infected with a retrovirus derived from pBabeHygro encoding the early region of SV40 (49) and selected with 100 μg/ml hygromycin to establish immortalized cultures. These immortalized MEFs, HEK-HT cells (4), and IMR90 (ATCC) human fetal lung fibroblasts (pd 38-40) were stably infected with retroviruses encoding no transgene or the indicated Ras genes, as previously described (49).

**RNA isolation and cDNA synthesis from urethane lung tumors.** Dissected tumors were homogenized and RNA isolated using RNA-Be
(Fisher Scientific). Phase-lock heavy gel (Fisher Scientific) maximized recovery. RNA was coprecipitated with Pellet Paint (Novagen) using isopropanol. Maximal RNA volumes were used for reverse transcription (50% of the total reaction volume) with M-MuLV reverse transcriptase (New England Biolabs), according to the manufacturer instructions, with the addition of Oligo(dT) primers and RNaseOUT.

**Infusion cloning of Kras amplicons and Sanger sequencing.** Primers with cloning adapters were used for PCR amplification of Kras (Supplemental Table 7). PCR amplification was performed using Platinum Taq polymerase, with the supplied buffer used at 0.7 times the suggested final concentration, 1 mM dNTPs, 2 mM MgCl₂, 0.4 μM forward and reverse primers, and 5% of the total reaction volume contributed by cDNA. Reactions were run for 40 cycles using the following standard conditions: denaturation at 94°C for 30 seconds, annealing at 55°C for 45 seconds, and elongation at 72°C for 2 minutes. Gel-purified PCR products were cloned into pBlueScript using In-Fusion HD (Stratagene), transformed into bacteria, and 8–12 Amp® clones per tumor sequenced using T3 and T7 primers as necessary to achieve insert coverage.

**Kras amplification and Ion Torrent PGM sequencing.** Primers with Ion Torrent PGM (Invitrogen) adapter sequences and barcodes corresponding to tumor sample and allele amplified (native or ex3op, Supplemental Table 7) were used for PCR amplification. PCR reactions used Platinum Taq, the supplied buffer (0.7 times final concentration), 1 mM dNTPs, 1 mM MgCl₂, 0.4 μM primers, and 5% of the total reaction volume contributed by cDNA. Reactions were run for 40 cycles as follows: denaturation at 94°C for 30 seconds, step-down annealing at 65°C to 50°C for 45 seconds, and elongation at 72°C for 2 minutes. 450-bp products were gel purified and pooled for sequencing. Sequencing reads were filtered to ensure high quality at the G12 codon, Q61 codon, and Kras-coding SNP. Usable reads were defined as exactly matching the consensus 4-bp region surrounding the G12 codon, Q61 codon, and Kras-coding SNP. Usable reads were defined as exactly matching the consensus 4-bp region surrounding the G12, Q61, and Kras coding SNP. Tumor sample datasets with at least 100 usable reads (representing >58% of total reads) or at least 600 usable reads (representing >45% of total reads) were evaluated for mutations. For these datasets, the 3-bp G12 and Q61 codon sequences and SNP were extracted from each usable read, and total counts for every observed G12, Q61, and SNP combination were quantified. For datasets derived from mixed-background Kras<sup>ex3op<sup>neo</sup></sup> mice, strain alleles were considered adequately sampled if representing greater than 16% of the usable reads in the dataset. Additionally, oncogenic mutations were called if they represented greater than 16% of the usable reads for a given allele (based on the frequency of detection in Sanger analysis).

To ensure that mutations were limited to the G12 codon, Q61 codon, and Kras-coding SNP, all reads passing the above filter were aligned to the Kras coding sequence reference spanning exons 1-3 using Bowtie2 and the following alignment parameters: -k 1 -N 1 -L 32 -local. Overall, we detected no other sequence differences within the first 200 bp (corresponding to the upper limit of accurate read sequencing via Ion Torrent) of the Kras cDNA. All genomic data are available in the NCBI’s SRA data repository (SRP041810).

**Growth arrest of primary human cells.** As previously described (23), IMR90 cells (pd 35-37) were infected with retroviruses derived from pBabePuro with no insert or the indicated HRAS or Kras oncogenes, selected with 2 μg/ml puromycin, and plated at 4,000 cells per well in 24-well dishes. On the indicated days, cells were fixed with 10% formalin and stained with 0.1% crystal violet (Sigma-Aldrich). For quantification, crystal violet was extracted with 10% acetic acid, and absorbance at 600 nm was normalized to day 1. Each comparison was performed at least twice, and each sample was evaluated at least in triplicate.

**Animal studies.** C57BL/6/J-CMV-Cre (50) and 129S/Sv-Prm-Cre (51) mice were obtained from The Jackson Laboratory. Kras<sup>ex3op</sup> chimeras were crossed with 129S6 mice and progeny screened for germline transmission by genotyping PCR (Supplemental Table 7). Following germline transmission, 129S6-Kras<sup>ex3op<sup>neo</sup></sup> mice were crossed with either B6.C-Tg(CMV-Cre)1Cgn/J or 129S/Sv-Tg(Prm-cre)8Bog/J mice, resulting in Cre-mediated excision of the neo cassette. Specific strains used for each experiment are detailed within the figures for clarity. Cohorts of mixed-background Kras<sup>ex3op<sup>neo</sup></sup> (n = 15) and littermate Kras<sup>ex3op<sup>nat</sup></sup> (n = 6) mice were monitored for over 18 months to evaluate for the development of abnormal phenotypes. Hras<sup>−/−</sup> and Nras<sup>−/−</sup> mice obtained from the National Cancer Institute (NCI) (52) were bred with Kras<sup>ex3op</sup> mice for assessment of progeny ratios. Pups were visually assessed for chylous ascites (14) between 3 and 8 days of age, followed by genotyping. CDKNA2a<sup>+/−</sup>, mice (53) were provided by Ron DePinho (Department of Cancer Biology, Division of Basic Science Research, The University of Texas MD Anderson Cancer Center, Houston, Texas, USA) and crossed with mice carrying the CMV-Cre transgene to generate whole-animal knockouts to cross with Kras<sup>ex3op</sup> mice.

**Urethane carcinogenesis.** Six- to 8-week-old mice were injected i.p. with urethane as previously described (18) and euthanized at the indicated time points indicated in Figures 2, 3, 5, and 7. Lung tumors were counted, measured, dissected and preserved in RNAlater (QIAGEN) or snap frozen in liquid nitrogen for sequencing analysis. Tumor burden was calculated as the sum of tumor diameters (mm). A subset of left lungs were fixed for histologic analysis.

**Statistics.** Statistical analyses were performed using GraphPad Prism software, version 6 (GraphPad Software). Unpaired, 2-tailed t tests with a 95% CI were used for 2-group comparisons. 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 χ² test was used for analysis of progeny ratios in breeding experiments. A P value of less than 0.05 was considered statistically significant.

**Study approval.** All mouse care and experiments were performed in accordance with protocols approved by the IACUC of Duke University.

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