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Report

Rare Codons Regulate KRas Oncogenesis

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Summary

Oncogenic mutations in the small Ras GTPases KRas, HRas, and NRas render the proteins constitutively GTP bound and active, a state that promotes cancer [1]. Ras proteins share ~85% amino acid identity [2], are activated by [3] and signal through [4] the same proteins, and can exhibit functional redundancy [5, 6]. Nevertheless, manipulating expression or activation of each isoform yields different cellular responses [7–10] and tumorigenic phenotypes [11–13], even when different *ras* genes are expressed from the same locus [6]. We now report a novel regulatory mechanism hardwired into the very sequence of *RAS* genes that underlies how such similar proteins impact tumorigenesis differently. Specifically, despite their high sequence similarity, *KRAS* is poorly translated compared to *HRAS* due to enrichment in genomically underrepresented or rare codons. Converting rare to common codons increases KRas expression and tumorigenicity to mirror that of HRas. Furthermore, in a genome-wide survey, similar gene pairs with opposing codon bias were identified that not only manifest dichotomous protein expression but also are enriched in key signaling protein classes and pathways. Thus, synonymous nucleotide differences affecting codon usage account for differences between HRas and KRas expression and function and may represent a broader regulation strategy in cell signaling.

Results

Oncogenic *HRAS* cDNA, when ectopically overexpressed in human cells, consistently produced 20-fold more protein than the identically tagged oncogenic version of the major splice form (4B) of *KRAS* cDNA (termed *KRAS*), regardless of the method of introducing DNA, cell type, epitope tag, promoter, exogenous UTRs, introns, mutations, or membrane targeting (Figure 1A; see also Figures S1A–S1H available online). To explore this difference further, we generated *KRAS** by fusing *HRAS* cDNA encoding the first 158 amino acids (mutated at nine residues to match KRas) to *KRAS* cDNA encoding the terminal hypervariable 30 amino acids (Figure S1I). *KRAS** produced more KRas protein than *KRAS*, regardless of other parameters, indicating that *KRAS* nucleotide sequence limits protein expression. *KRAS** still expressed less protein than *HRAS* (Figures 1A, S1A–S1C, and S1E–S1G); whether this is a consequence of the remaining *KRAS* sequence or is due to other mechanisms remains to be determined. Additionally, pERK and pAKT levels, measures of Ras effector activation, were similar in cells expressing oncogenic HRas, KRas, or KRas* (Figure S1G). Brief metabolic labeling revealed higher KRas protein expression from *KRAS** compared to *KRAS* (Figure S1J), consistent with a translational etiology. Indeed, glutathione S-transferase (*GST*) fused with *KRAS* cDNA (GST-KRas) expressed little protein unless a STOP codon was inserted between the two (GST-STOP-KRas) or KRas protein was encoded from *KRAS**, suggesting that ribosomes stall upon entering *KRAS* mRNA (Figure 1B). Moreover, *KRAS* and *KRAS** mRNA levels were within 2-fold of *HRAS* mRNA (Figure S1K), although *KRAS* was consistently the lowest, perhaps reflecting no-go decay of stalled transcripts [14]. Thus, the nucleotide sequence of *KRAS* message impedes translation.

Comparing the *KRAS* and *HRAS* sequence revealed that the third position of codons was typically an A/T in *KRAS* but G/C in *HRAS*. Based on human genome-wide analysis of the relative frequencies that degenerate codons are used to encode for the same amino acid [15], the A/T bias in *KRAS* corresponds to underrepresented (rare) codons (Figure 1C). Consistent with rare codons limiting protein expression, KRas levels were increased when translated from *KRAS** mRNA (Figure 1A). Similarly, the bias toward rare codons was conserved in mammalian and avian *KRAS* genes (Figure S1L) and equated with lower expression compared to HRas in mammalian cells (Figure 1D). Moreover, zebrafish *hras* and *kras* encoded by a mixture of rare and common codons exhibited comparable protein expression at a level between that of mammalian HRas and KRas (Figures 1D and S1L). Finally, progressively converting rare to common codons proportionally increased human KRas protein expression, even upon changing only nine rare isoleucine and valine codons scattered throughout *KRAS* to their common counterparts (Figures 1E and S1I). Thus, rare codons limit *KRAS* translation.

To investigate this phenomenon at the endogenous level, we infected HCT116 human cancer cells (which have a $KRAS^{G13D}$ allele) with an AAV targeting vector [16] designed to knock into *KRAS* exon 1 either $opKRAS^{G13D}$ (oncogenic $KRAS^{G13D}$ cDNA in which 130 rare codons were *op*timized to common codons) or $uaKRAS^{G13D}$ (*ua*ltered $KRAS^{G13D}$ cDNA). The resultant transcripts would be expressed from the endogenous *KRAS* promoter and retain the 5′ UTR and first 17 coding nucleotides, minimizing effects on translation initiation, but encode only the *KRAS4B* spliced product (Figure 2A). Screening ~1,800 neomycin-resistant clones revealed four with $opKRAS^{G13D}$ and two with $uaKRAS^{G13D}$ cDNA successfully knocked into one allele of the *KRAS* gene. Immunoblot revealed poor expression of endogenous KRas in both the $uaKRAS^{G13D}$ and control cell lines exhibiting a random integration event, on par with the parental HCT116 cells. Conversely, the $opKRAS^{G13D}$ cell lines exhibited on average 5-fold higher KRas protein and 2-fold higher *KRAS* mRNA levels (Figures 2B, S2A, and S2B). This increase was lower compared to the ectopic setting, consistent with overexpression systems magnifying the effects

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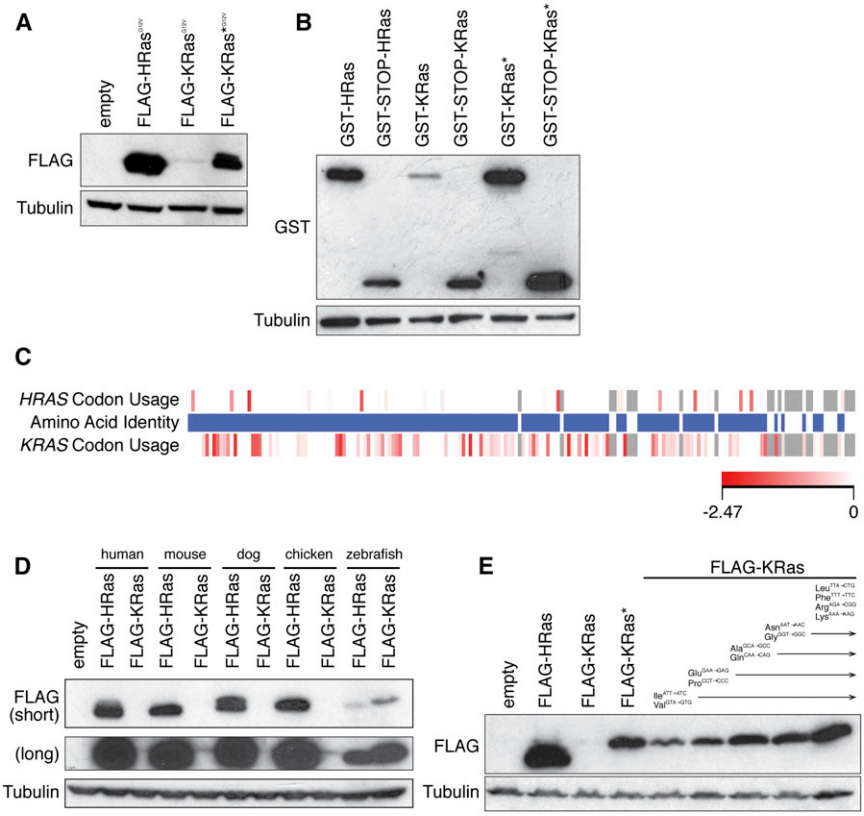

Figure 1. Translation of *KRAS* Is Limited by Rare Codons

(A) Immunoblot of lysates isolated from human HEK-HT cells stably infected with the retrovirus pBABEpuro encoding the indicated N-terminal FLAG epitope-tagged oncogenic (G12V) human Ras proteins (FLAG-Ras^{G12V}) with an α-FLAG or α-tubulin antibody. One of three experiments is shown.

(B) Immunoblot of lysates isolated from HEK-HT cells transiently transfected with the plasmid pCIneo encoding *GST* cDNA, a STOP codon where indicated, and in frame the indicated FLAG-Ras cDNAs, with an α-GST or α-tubulin antibody. One of three experiments is shown.

(C) Amino acid identity of HRas and KRas is shown in the middle bar. Blue indicates identical amino acids; white indicates nonidentical amino acids. Relative codon usage of *HRAS* versus *KRAS* is shown in the upper and lower bars, respectively. Increasingly dark shades of red indicate increasing relative rarity of the codon for identical amino acids; gray indicates gaps in alignment or nonidentical amino acids.

(D) Immunoblot of lysates isolated from human HEK-HT cells stably infected with the retrovirus pBABEpuro encoding the indicated FLAG-Ras proteins isolated from the indicated species with an α-FLAG or α-tubulin antibody. One of two experiments is shown.

(E) Immunoblot of lysates isolated from human HEK-HT cells stably infected with the retrovirus pBABEpuro encoding FLAG-HRas, FLAG-KRas, or FLAG-KRas* (in which the indicated rare codons were progressively converted to the indicated common codons) with an α-FLAG or α-tubulin antibody. One of two experiments is shown.

Detailed methodologies and reagent descriptions are provided in Supplemental Experimental Procedures. See also Figure S1.

of codon bias [17]. Thus, changing rare to common codons increases endogenous KRas protein expression.

Polysome profiling was performed to characterize relative ribosomal kinetics of endogenous *HRAS* and endogenous untargeted *KRAS* mRNAs from HCT116 clone 5. Semiquantitative RT-PCR revealed that both transcripts accumulated in polysome fractions, although *KRAS* occupied relatively heavier fractions despite nearly equal transcript length (Figure 2C), indicating more dense packing of ribosomes. Pactamycin treatment to halt translation initiation (Figure S2C) led to an accumulation of *HRAS* mRNA in lighter polysome and ribosome-free fractions, indicative of ribosome translocation. As observed previously [18], there was only a minimal shift of *KRAS* mRNA in the gradient, with the bulk of the message retained in the heavy fractions (Figure 2C). These results were confirmed in HEK-HT cells (Figure S2D). Polysome profiles of mRNA derived from the *opKRAS*^{G13D} HCT116 clone 5 and *uaKRAS*^{G13D} HCT116 clone 1 were compared. Quantitative RT-PCR analysis revealed that after pactamycin treatment (Figure S2C), *opKRAS*^{G13D} mRNA was modestly shifted to lighter fractions relative to *uaKRAS*^{G13D} mRNA (Figure 2D), consistent with the degree that other transcripts are shifted upon alleviating ribosome stalling [19]. These shifts were recapitulated in *opKRAS*^{G13D} HCT116 clone 4 (Figures S2C and S2E). Thus, rare codons impede translation of endogenous *KRAS* mRNA.

The biological impact of codon bias in mammalian genes is largely unknown. However, targeted replacement of codons in the *Drosophila* alcohol dehydrogenase gene with either suboptimal [20] or optimal [21] codons can respectively decrease or increase enzyme activity and alcohol tolerance, suggesting that codon bias affects gene function in higher eukaryotes. Because oncogenic Ras can impart tumorigenic growth to cells, we used tumor growth to assess the biological impact of altering codon bias of human *KRAS*. HEK-HT cells, which require oncogenic HRas for tumor growth [22], were transduced with a vector expressing no transgene, oncogenic HRas^{G12V}, KRas^{G12V}, or KRas*^{G12V} (Figure 1A) and assayed for tumor growth. KRas^{G12V}, like vector cells, formed tumors with 90-fold smaller masses and reduced kinetics compared to HRas^{G12V} cells, an effect rescued by *KRAS**^{G12V} (Figures 3A–3C). Tumors that eventually formed 3 months later from KRas^{G12V} cells exhibited elevated KRas expression (Figure S3A). To examine the endogenous effect, we compared *opKRAS*^{G13D} and *uaKRAS*^{G13D} knockin clones in which targeted recombination occurred at the same wild-type *KRAS* allele. *opKRAS*^{G13D} clone 5 formed tumors with up to 30-fold larger masses and increased kinetics compared to *uaKRAS*^{G13D} clones (Figures 3D–3F), although this was not as dramatic as observed in the ectopic setting. Tumors from *opKRAS*^{G13D} cells retained differential KRas protein expression and, interestingly, exhibited higher HRas expression (data not shown). Although altering rare codons may affect protein folding and processing [17], there is a direct correlation among codon content, protein expression, and tumorigenesis of ectopic and endogenous oncogenic *KRAS*.

To explore whether the differences in codon bias between *RAS* genes reflect a broader regulation strategy, we performed a genome-wide survey to identify gene pairs with high similarity and divergent codon usage (Table S1) using G-C content

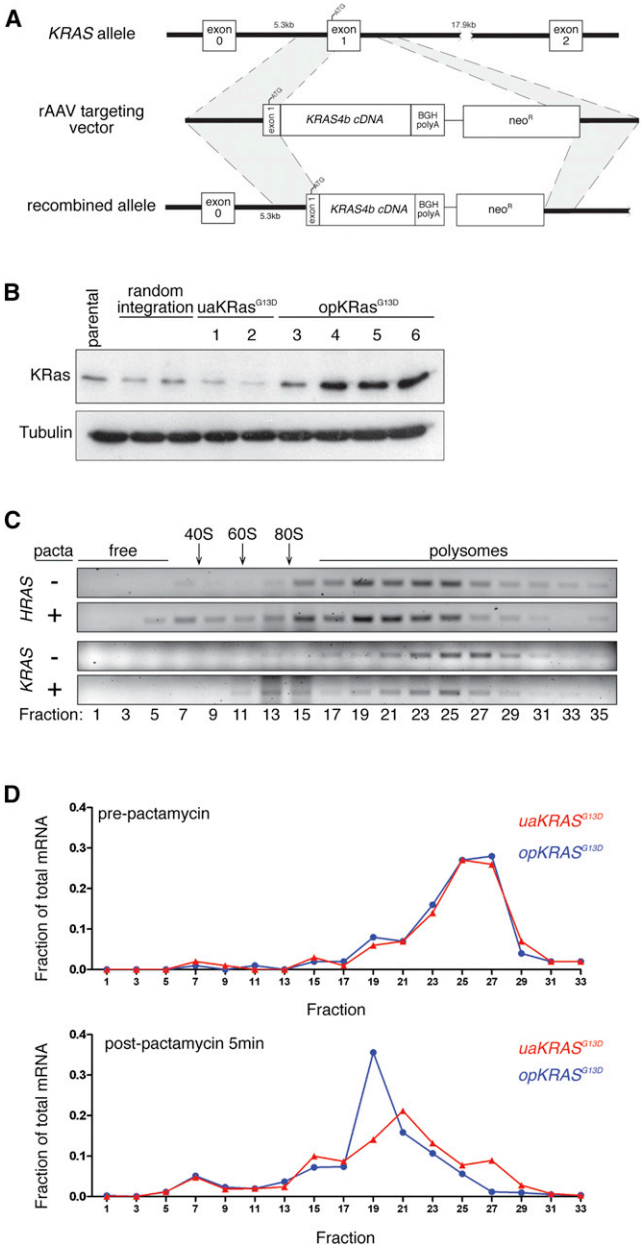

Figure 2. Rare Codon Bias Limits Endogenous *KRAS* Translation

(A) AAV-based recombination targeting strategy to knock into exon 1 of the endogenous *KRAS* gene in human HCT116 colon cancer cells (ATCC) an oncogenic $KRAS^{G13D}$ cDNA in which either 130 rare codons were *op*timized to common codons ($opKRAS^{G13D}$) or left *u*naltered ($uaKRAS^{G13D}$) [16].

(B) Immunoblot of lysates isolated from the parental HCT116 cell line, stable clones with nonhomologous vector integration, clones with homologous integration of $uaKRAS^{G13D}$, and clones with homologous integration of $opKRAS^{G13D}$, with an α-KRas or α-tubulin antibody. Data shown are representative of at least one experiment.

(C) Semiquantitative RT-PCR using primers specific for *HRAS* and the nontargeted *KRAS* mRNA of the indicated sedimentation fractions (relative positions of ribosome-free, ribosome subunits 40S, 60S, and 80S, and polysome fractions) isolated from HCT116 $opKRAS^{G13D}$ clone 5 pre (−) and post (+) pactamycin (pacta) treatment to halt translation initiation.

(D) Quantitative RT-PCR using primers specific for the $KRAS^{G13D}$ knockin mRNA of the indicated sedimentation fractions isolated from HCT116 $opKRAS^{G13D}$ clone 5 and $uaKRAS^{G13D}$ clone 1 pre and post pactamycin treatment to halt translation initiation.

Detailed methodologies and reagent descriptions are provided in Supplemental Experimental Procedures. See also Figure S2.

at synonymous sites (GC3) as a proxy for rare codons [15]. Using *KRAS* and *HRAS* as a benchmark, the top 60 gene pairs had ≥80% amino acid identity and ≥1.8-fold difference in GC3 content (Figure 4A; Table S1). Twelve such cDNA pairs were Myc epitope tagged and expressed in human cells. In every case, the gene enriched in rare codons expressed less protein. Changing rare to common codons in the two genes *CFL2* and *ORMDL1* augmented protein expression to that of *CFL1* and *ORMDL3* (Figure 4B). Thus, synonymous differences altering codon usage influence expression of mammalian genes encoding similar proteins.

To assess the functional significance of this gene set, we performed gene ontology analysis [23] on the top 150 gene pairs sharing high identity and large differences in GC3 content (Table S1). Strikingly, we found that this set was highly enriched for proteins with purine nucleotide binding or kinase activity. This enrichment was six or more orders of magnitude more significant than enrichment of any other functional category from the analysis of gene pairs from complementary combinations of identity and GC3 content criteria (Figure 4C; Table S2). KEGG pathway analysis [24] was also performed to determine whether gene pairs reside in pathways. Insulin signaling, long-term potentiation, and tight junction pathways, in particular, were significantly enriched for gene pairs with high protein identity and differential codon usage (Figure 4D; Table S3). Thus, the combination of two seemingly unrelated criteria—high protein identity and opposing codon bias—identified unique functional classes of proteins as well as signaling pathways.

Discussion

Although differences in KRas and HRas expression have been previously reported in at least some settings [25], the mechanism responsible and effects thereof remained unknown. Here we present a novel regulatory strategy that has been hiding in plain sight: codon bias. Rare codons throughout *KRAS* message impede translation and correspondingly protein, and to a lesser extent mRNA levels, and reduce oncogenic activity. This was surprising because, although rare codons impede protein translation in heterologous expression systems [26, 27] and common codon bias exists in highly expressed genes in bacteria and yeast [17], there are few reports of it affecting ectopic expression of homologous transcripts [28–30], let alone endogenous mammalian genes. Moreover, codon bias in mammals has been argued to be a by-product of genes residing within larger genomic regions of nucleotide bias (isochores) that affect chromatin functions like meiotic recombination [31]. Furthermore, in genome-wide translational assessment, sites of ribosome stalling do not correlate with presence of rare codons [32]. Nevertheless, the effects of codon usage between *RAS* genes on protein expression and function suggest that there are indeed consequences resulting from synonymous differences at the nucleotide level [20, 21, 33].

At face value, the finding that *KRAS* is an incredibly weak oncogene due to rare codon bias seems at odds with this gene being the most commonly mutated *RAS* isoform in cancer [34]. However, untransformed cells are sensitive to oncogenic stress [35], and increasing levels of transgenic $Hras^{G12V}$ result in progressively more growth-arrested (senescent) cells and fewer mammary lesions in mice [36]. Perhaps rare codons limit the expression of KRas to

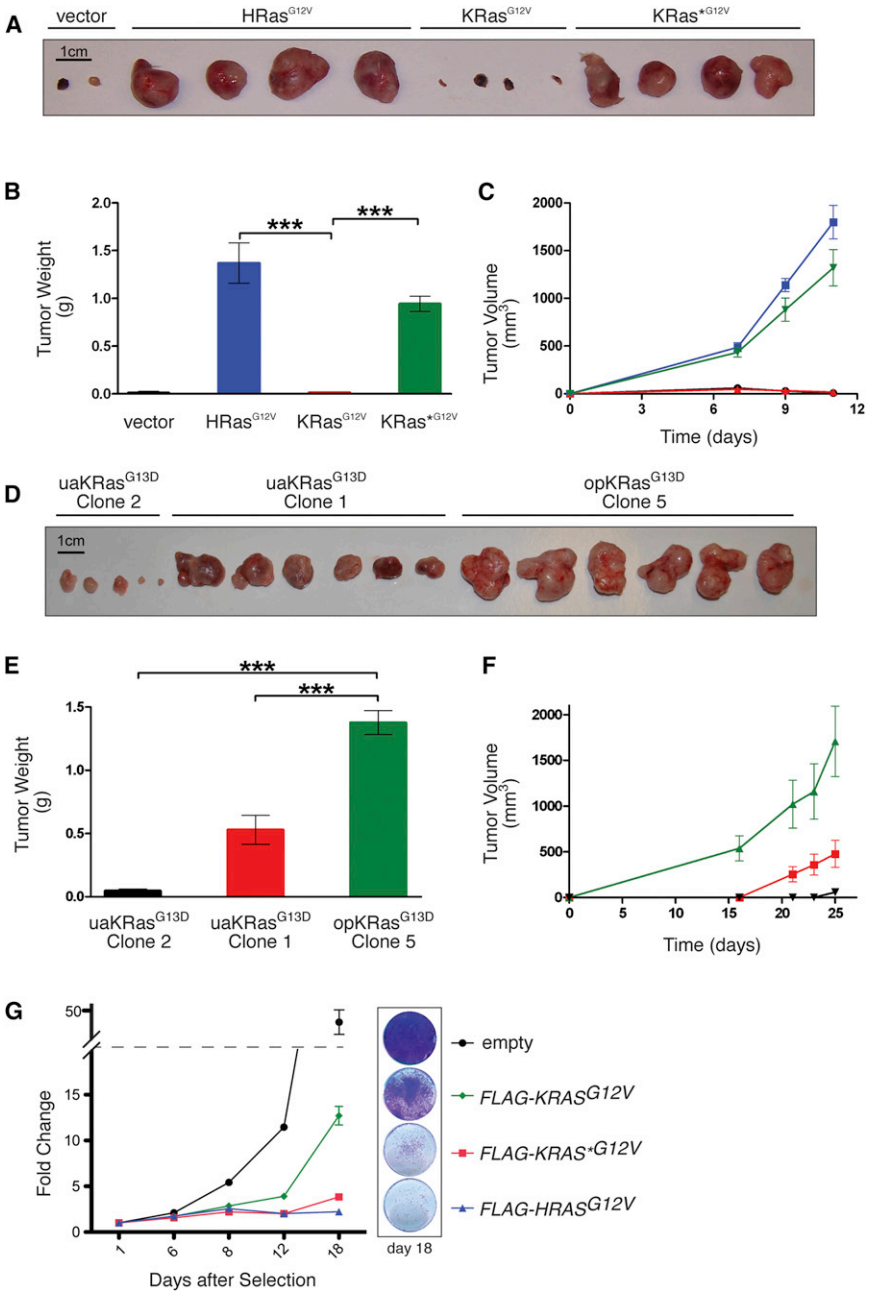

Figure 3. Rare Codons Limit Oncogenic KRas-Driven Tumorigenesis

(A–C) Photograph (A) and mean weight ± SEM (B) of tumors at endpoint, as well as mean size ± SEM of tumors over time (C) derived from HEK-HT cells stably expressing empty vector (black circles), HRas^{G12V} (blue squares), KRas^{G12V} (red triangles), and KRas^{*G12V} (green triangles) (n = 4). One of two experiments is shown. ***p < 0.001.

(D–F) Photograph (D) and mean weight ± SEM (E) of tumors at endpoint, as well as mean size ± SEM of tumors over time (F) derived from HCT116 clones expressing opKRas^{G13D} (clone 5; green triangles), uaKRas^{G13D} (clone 1; red squares), or uaKRas^{G13D} (clone 2; black triangles) (n = 6). One of two experiments is shown. ***p < 0.001.

(G) Change in IMR90 cell number over time following acute expression of the indicated FLAG-Ras^{G12V} constructs. Left: fold change in crystal violet absorbance at 590 nm at the indicated time points after cell plating is normalized to day 1 for each cell line. Relative cell number for IMR90 cells expressing any of the oncogenic FLAG-Ras constructs was significantly lower than empty vector control cells on days 8, 12, and 18 ($p < 0.01$). Additionally, relative cell numbers for both empty vector and *FLAG-KRAS^{G12V}*-expressing cells were significantly different from each other and *FLAG-KRAS^{*G12V}* and *FLAG-HRAS^{G12V}* cells on days 12 and 18 ($p < 0.0001$). Right: crystal violet staining of intact cells at day 18, representative of four replicates. Data shown are representative of at least one experiment.

Detailed methodologies and reagent descriptions are provided in Supplemental Experimental Procedures. See also Figure S3.

an ideal range to initiate tumorigenesis—high enough to promote hyperplasia but low enough to avoid excessive senescence. Indeed, IMR90 primary human fibroblasts expressing *KRAS^{*G12V}* or *HRAS^{G12V}*, but not *KRAS^{G12V}*, appeared to arrest with a senescent morphology (Figure 3G; data not shown). Interestingly, there is concordance between the degree of rare codon bias and the mutation frequency among *RAS* family members (Figures S3B and S3C). However, codon bias cannot alone account for this difference, because *Hras* cDNA knocked into the *Kras* locus is mutated at a high frequency in a urethane model of lung cancer [6]. Nevertheless, rare codons clearly crippled the oncogenic activity of *KRAS*, which could be a barrier to malignant progression. In this regard, it has been hypothesized that upregulation of oncogenic KRas is a necessary intermediate step in tumor progression after senescence escape [36]. Multiple mechanisms that may increase KRas expression occur during cancer, including *KRAS* gene amplification [37, 38], microRNA downregulation directly [39, 40] or indirectly by sequestration via *KRAS* pseudogene mRNA [41], and a general increase in tRNA levels [42, 43].

It was also striking that the divergent tumorigenesis phenotypes of two nearly identical proteins were reconciled by altering codon usage. It is formally possible that changing codon bias affected some other aspect of mRNA regulation, but the multitude of complementary codon modification strategies implemented make this possibility less likely. Codon usage might underlie functional differences between members in other protein families with opposing codon bias. The enrichment of gene pairs with high amino acid sequence identity and divergent codon bias in signaling networks further suggests that codon usage may impact entire signaling pathways. Indeed, differential regulation of functionally redundant genes may add specificity to signal transduction [44], and there are situations in which transcripts enriched in rare codons are preferentially translated in mammalian cells [45]. Thus, codon bias is not only a novel mechanism regulating Ras isoforms but may reflect a broader regulatory strategy in signaling pathways.

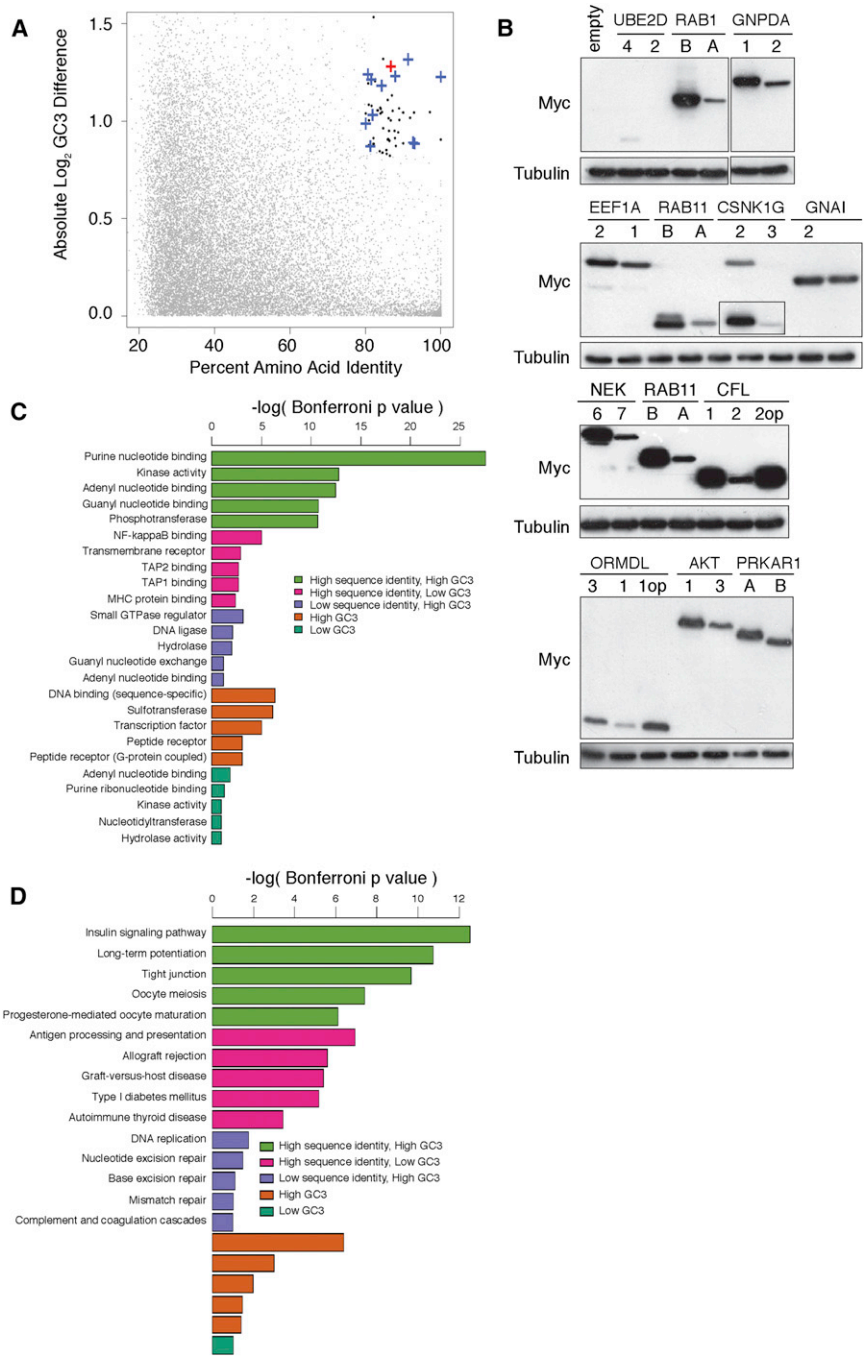

Figure 4. Gene Pairs with Divergent Codon Bias Demonstrate Correlating Differences in Expression and Cluster in Unique Signaling Protein Classes

(A) Percent amino acid identity versus log difference in CDS GC3 content of individual protein pairs identified by BLAST alignment (gray points). Black points: gene pairs with $\geq$80% identity and $\geq$1.8-fold difference in GC3 content; blue crosses: gene pairs tested for protein expression; red cross: *HRAS-KRAS* gene pair.

(B) Immunoblot of lysates isolated from human 293 cells (ATCC) stably infected with the retrovirus pBABEpuro encoding the indicated N-terminal FLAG epitope-tagged cDNAs corresponding to human gene pairs of high amino acid sequence identity that have a common (first) versus a rare (second) codon bias, or the gene pair enriched in rare codons after rare codons were optimized (op) to common codons, with an α-FLAG or α-tubulin antibody. Data shown are representative of at least one experiment.

(C and D) Histogram comparing p values of gene ontology categories (C) or KEGG signaling pathways (D) enriched in lists of gene pairs with high amino acid sequence identity and high GC3 difference (green), high identity and low GC3 difference (pink), low identity and high GC3 difference (purple), or lists of genes with high GC3 content (brown) or low GC3 content (aquamarine).

Detailed methodologies and reagent descriptions are provided in Supplemental Experimental Procedures. See also Tables S1, S2, and S3.

Supplemental Information

Supplemental Information includes three figures, Supplemental Experimental Procedures, and three tables and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2012.11.031.

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